OPTIMIZATION OF AMINO ACIDS MEASUREMENT AND EVALUATION OF JUICE BLENDS FOR ENHANCED HEALTH-PROMOTING PROPERTIES IN SELECTED VEGETABLES

A Thesis

by

KAREN ANDREA CORLETO

Submitted to the Office of Graduate and Professional Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

Chair of Committee,	Bhimanagouda S. Patil
Committee Members,	G.K. Jayaprakasha
	Richard Kreider
Head of Department,	Boon Chew

December 2017

Major Subject: Nutrition

ABSTRACT

Amino acids are involved in numerous biological processes such as protein synthesis, cell signaling, and immune response, and some act as antioxidants. In the first study, a rapid, sensitive and reproducible HPLC-FLD method was developed for the quantification of amino acids including L-citrulline from vegetables juices and commercial juices. The method separated 21 amino acids using an octylsilyl stationary phase. Optimal separation conditions were obtained with 20 mM sodium acetate buffer (solvent A) and water with organic modifier acetonitrile and methanol (solvent B). The developed method was validated with six vegetable juices: watermelon, cucumber, celery, calabaza squash, zucchini squash, yellow squash and commercial juice samples. Results demonstrated that L-citrulline content was highest in watermelon juice.

Nitrate and polyphenols from the diet may enhance the production and bioavailability of nitric oxide, a radical signaling molecule essential for cardiovascular health. In the second study, the stability of nitrate and total phenolics in beetroot and arugula juices was measured over one month at different temperatures. The levels of nitrate were highest at the initial day samples for beetroot and arugula juices. At 25 °C nitrate degradation initiated within 24 h, whereas nitrate remained stable in frozen samples for one month. UPHLC-HR-QTOF-MS was applied for the identification of bioactive compounds such as betacyanins, polyphenols and their degradation products from beetroot and arugula juices.

In the third study, the juices of watermelon, beetroot, kale, arugula, and six different juice blends were evaluated for their bioactive composition, amino acid profile and antioxidant activity. Results demonstrated that beetroot juice had the highest level of nitrate followed by kale and arugula. For the blends, B-1 and B-2 had significantly higher levels of nitrate than the other juice blends. Watermelon juice was found to have the highest levels of L-citrulline followed by blend B-6. The juices were also freeze-dried and then evaluated for their nitrate and L-citrulline content. In conclusion the blending of vegetable juices can increase the levels of bioactive compounds and enhance the nitrate and L-citrulline content, which may lead to improved cardiovascular health and sports performance.

DEDICATION

In memory of my grandfather Edgar Carvajal; you taught me that the puzzles in life will always come together with a little effort and that they are sunnier when you have someone by your side. You saw the start of my journey and I will carry the memories and lessons you shared with me to the end, till we meet again.

ACKNOWLEDGEMENTS

I would like to thank my committee chair, Dr. Bhimanagouda Patil, and my committee members, Dr. Jayaprakasha, G.K., and Dr. Richard Kreider, for their guidance and support throughout the course of this research. Their passion and drive towards learning and research is inspiring and I will always strive to do the same. I am very grateful for the knowledge that I have gained during the course of this degree under their guidance.

I would also like to express my heartfelt thanks and gratitude to all my colleagues at Texas A&M University for enriching my experience. Without their friendship and support this degree would not have been possible; a special mention to Dr. Singh, Pratibha, Ripan and Siddu.

I would like to thank my loving parents for giving me a strong moral, spiritual, and educational background and up bring that has shaped me into the person I am becoming. Thank you for teaching me the value of education by being examples and by giving me the best education possible from homeschooling through college. Thank you for all the sacrifices that both of you have made for me and my siblings. Dad thank you for always challenging me try harder than what I thought possible and for your guidance and prayers. Mom, thank you for all your love, support, and heartfelt prayers. Without the constant love and support both of you have poured out on me since the beginning none of my dreams would have become accomplishments.

Carla and Josue thank you for your prayers, love and support. Thank you for reminding me to relax and enjoy life. Gig'em Aggies! To the 3 Aggies mom and dad brought up.

Most importantly, I would like to thank God who grants all blessings and gives abundantly. Thank you for the opportunity to pursue my dreams, the strength to accomplish them, and giving me a loving family to support me.

CONTRIBUTORS AND FUNDING SOURCES

Contributors

This work was supervised by a thesis committee consisting of Professor Bhimanagouda S. Patil the committee chair of the Department of Nutrition and Food Science, Professor G.K. Jayaprakasha of Department of Horticultural Sciences and Dr. Richard Kreider of the Department of Health & Kinesiology.

All work for the thesis was completed independently by the student.

Funding Sources

This project was supported by the Agriculture and Food Research Initiative competitive grant 2014-70003-22356 and 2015-67013-22813 and of the USDA National Institute of Food and Agriculture.

TABLE OF CONTENTS

Page

LIST OF FIGURES

FIGURE Page

LIST OF TABLES

TABLES Page

CHAPTER I

INTRODUCTION

Fruits and vegetables are considered an integral part of a healthy diet and are good sources of vitamins (A, B-complex, E, C, K etc.), minerals, potassium, nitrate, amino acids, fiber, and phytochemicals.[1-3] Phytochemicals are plant secondary metabolites involved in plant defense and various other functions.[4] Phytochemicals such as carotenoids, anthocyanins, flavonoids, phenolics, and other bioactive compounds contribute to many of the human health benefits associated with fruits and vegetables.[2, 3] Cardiovascular disease and cancer are leading causes of death in the United States and westernized nations, and epidemiological evidence suggests that increased consumption of fruits and vegetables may lead to a reduced risk for chronic diseases.[1, 2, 5, 6] Plant foodstuffs, and consumption and development of functional foods have become the focus of much research.[1-3, 7-10] Functional foods, whole foods or food products that contain significant amounts of bioactive compounds, can also be used as ergogenic aids as a natural way to enhance sports performance.[11-14]

Fruit and vegetable juices can be considered functional foods and provide a vehicle to increase fruit and vegetable consumption. Beverages with functional properties are popular due to their ease of consumption and it is possible to incorporate beneficial compounds to maximize their health benefits. Recently, many sports-related ergogenic aids and health related supplements have been packaged as beverages.[13, 14] Furthermore, fresh fruit juices and smoothies can be used as natural additive-free sports aids. Accumulating evidence suggests that amino acids, dietary nitrate, polyphenols,

ascorbic acid, and other bioactive compounds in fruits and vegetables not only a play role in human health but also enhance sports performance.[7, 11, 14-17]

Nitric oxide, a free radical compound produced *in vivo* has various effects on the body.[18] One of the important effects is signaling for vasodilation that allows improved blood flow. These effects allow for the potential to improve cardiovascular health in pre-hypertensive and hypertensive individuals and sports performance. Two pathways leading to the production of nitric oxide are the nitric oxide synthase (NOS)-dependent and NOS-independent pathway, which use L-arginine/L-citrulline and dietary nitrate, respectively.

Certain selected fruits and vegetables are excellent sources of L-arginine and L-citrulline and dietary nitrate. Recent studies have demonstrated that beetroot juice containing dietary nitrate improves blood flow by causing vasodilation via supplementation to the NOS-independent pathway, also called the nitrate-nitrite-nitric oxide pathway.[19-21] This pathway produces nitric oxide by the reduction of dietary nitrate to nitrite by oral bacteria in the oral cavity; this nitrite is later reduced to nitric oxide. L-citrulline and L-arginine from watermelon and other sources also improved vasodilation through the NOS-dependent pathway, also called the L-arginine-Nitric Oxide Pathway.[12, 22] Nitric oxide is produced endogenously by inter-conversion of L-arginine to L-citrulline via the enzyme NOS in this pathway.

Considering that vasodilation improves sports performance, it is possible that certain bioactives from selected fruits and vegetables can potentially lead to the production of nitric oxide that may have a positive impact on sport performance. Further

research on the effects of functional foods and beverages, and these selected compounds, is needed to develop natural products from fruits and vegetables. These products containing bioactive compounds from fruits and vegetables which may potentially have synergistic effects may benefit cardiovascular health and sports performance.

Bioactive compounds have demonstrated health effects and sports performance enhancement, but the bioavailability of compounds such as L-citrulline and nitrate will determine their overall effectiveness. Dietary nitrate showed good bioavailability when consumed in raw and cooked vegetables, with almost 100% plasma bioavailability in some cases.[23] Studies have also shown positive nitrogen oxide (nitrate plus nitrite) bioavailability after consumption of beetroot juice and whole beetroot.[24, 25] Supplementation with L-citrulline has shown a significant increase in plasma citrulline and arginine levels.[26, 27] It was also found that in caco-2 cells, citrulline has higher bioavailability from natural watermelon juice compared to pasteurized watermelon juice and citrulline standard.[12] These findings suggest that the food matrix plays a role in the bioavailability of L-citrulline.

Due to the potential health and sports performance-enhancing benefits attributed to nitric oxide, the purpose of this study was aimed at developing functional vegetable juice blends with enhanced L-citrulline and nitrate content. The following objectives were hypothesized for the thesis.

- To analyze nitrate and nitrite in vegetable juices by high performance liquid chromatography (HPLC) and determine short-term and long-term stability in fresh beetroot and arugula juices.

- To develop an optimized pre-column derivatization HPLC-FLD method for analysis of free amino acids in vegetables juices.
- To determine the ideal blend of vegetable juices from beetroot, watermelon, arugula, and kale for optimal nitrate, amino acids, and antioxidant activity.

CHAPTER II

REVIEW OF LITERATURE

2.1 Selected Vegetables

2.1.1 Beetroot

Beetroot is an edible taproot that is widely consumed. [28] Since beetroot contains various beneficial compounds, including dietary nitrate, betalains, hydroxycinniamic acids, and flavonoids, it has recently received attention as a functional food. [28, 29] Compared with whole roots, beetroot juice seems to be a better source of dietary polyphenols due their higher bio accessibility. [11] Ingestion of beetroot juice increased nitrite plasma levels due to its dietary nitrate content. [30] Accumulating evidence suggests vasoregulatory effects of beetroot consumption because of its nitrate content, which may have potentially beneficial implications for cardiovascular health and sports performance.[19-21, 29, 30]

2.1.2. Arugula

Arugula, also known by various other names such as rocket, rucola, and roquette, is a green leafy vegetable. [31] Before emerging in the western diet, arugula first originated in Mediterranean and Middle Eastern countries. It has a distinctive, peppery taste and pungent aroma. [31, 32] Arugula is part of the Brassicaceae family with the main species being *Eruca sativa* and *Diplotaxis tenuifolias.* [33] It is a fast growing, cool weather crop with a relatively long storage and shelf-life in comparison with other leafy greens. [31, 34, 35] Arugula contain high fiber, vitamin C, and nitrate, and possesses many phytochemicals, such as carotenoids, glucosinolates, and flavonoids. [31-34, 36-38] Several

health benefits of arugula have been demonstrated, including antiplatelet, antidiabetic, antithrombotic, anti-inflammatory, anti-ulcer, anti-cancer, and hepatoprotective effects. [31-36, 38] Arugula accumulates high levels of nitrate, which seems to have cardioprotective properties and may aid in blood pressure regulation. [8, 39, 40] Hetta et al. found that certain extracts from *Eruca sativa* had antidiabetic properties. [38] Furthermore, antiplatelet and antithrombotic activities were also found in arugula extracts. [34] Considering its multitude health benefits due to the diverse phytochemical profile, arugula, can potentially be used to improve health and wellbeing. However, due to its pungent aroma, consumption of larger quantities of arugula would be a challenging task.

2.1.3. Watermelon

Watermelon, a cucurbit, is considered as a vegetable by its classification in the United States; with Florida, Georgia, California, and Texas being the top producing states. [41] It is popularly consumed mainly as a dessert food due to its sweetness. [41] Watermelon contains mineral salts (K, Mg, Ca and Fe), vitamins (A, B, C and E), amino acids including L-citrulline and L-arginine, carotenoids, and phenolics [41-43]. It is a good source of the carotenoid lycopene. Recent research suggests that fresh watermelon juice increases blood plasma *cis* and *trans* lycopene and β-carotene levels similar to thermally processed tomatoes.[44] Consumption of watermelon juice, a rich source of the amino acid L-citrulline, which is a precursor for L-arginine, increases plasma levels of the amino acid L-arginine in adults. [18, 27, 45]

2.1.4. Kale

Kale, a leafy green, is part of the Brassicaceae family, specifically *Brassica oleracea*, which also includes cabbage, broccoli, cauliflower, and Brussels sprouts. Kale is commonly cultivated in the United States, and Central and Northern Europe and grows as a biannual that tolerates a vast range of climatic and agricultural conditions.[46,47] It contains a broad range of naturally occurring compounds, including glucosinolates, vitamins, polyphenols, phenolic acids, chlorophylls, carotenoids, and amino acids.[46-54] Brassicas are a significant source of vegetable protein.[51] Varying levels of amino acids have been reported; however, most conclusively show glutamic acid to be the dominant amino acid.[50, 51] High levels of the glucosinolate sinigrin have been reported in kale.[48]

2.2 Dietary Nitrate Metabolic Pathway and Relevance

2.2.1. Dietary nitrate

Nitrate, NO_3^-, is a common relatively non-toxic inert inorganic anion found naturally in water, soil, and food.[8, 39] Vegetables are considered the main sources of dietary nitrate, reportedly contributing ~85% of nitrate.[8] Levels of dietary nitrate vary with different crops. For example, leafy greens, beetroot, and radishes are considered rich sources, but onions and garlic have lower levels.[8] Factors that influence the accumulation of nitrate in vegetables are genotype, temperature, sunlight, and fertilization.[8, 35] For biological activity, reduction to nitrite (NO_2^-) is essential; but issues regarding safety and toxicity of nitrate should also be considered due to its conversion to nitrite and other potential metabolites such as nitrosamines. Methemoglobinemia, "blue baby syndrome", has been linked to nitrate and nitrite consumption.[55] Unsanitary

conditions, yielding bacterial conversion of nitrate into nitrite in water, may be a potential cause for methemoglobinemia. Evidence for cancer caused by nitrosamine production is inconclusive and needs further investigation. Due to these concerns of toxicity, regulations have been placed on the acceptable levels of nitrate in water, fruits, and vegetables. The Accepted Daily Intake (ADI) for consumption of nitrate and nitrite were established by the European Food Safety Authority respectively as (0–3.7mg/kg) and (0-0.06mg/kg).[8] However, recent research has started to change the negative perception of dietary nitrate. Evidence suggests consumption of dietary nitrate in vegetables and fruits may have health benefits attributed to the conversion of nitrate to the signaling molecule nitric oxide.

2.2.2. Nitrate-nitrite-nitric oxide pathway

Production of nitric oxide via dietary nitrate is initiated by reduction to nitrite in the oral cavity by facultative anaerobic bacteria via nitrate reductase.[8, 39] After reduction, nitrite is swallowed and then converted to nitric oxide in the acidic conditions of the stomach and other parts of the body.[8, 30, 39] The conversion of nitrate to nitrite in the oral cavity through oral bacteria is crucial for the nitrate-nitrite-nitric oxide pathway to be effective. Govoni et al. found that the conversion of nitrate to nitrite is directly dependent on bacterial conversion in the mouth.[56] Rinsing with mouthwash reduced the amount of nitrate converted to nitrite.[56]

2.2.3 Dietary nitrate in sports performance and cardiovascular health

Dietary nitrate, as a sports supplement, may increasingly play an important role in exercise performance as research continues to demonstrate its effects in this area.

Supplementation with dietary nitrate was found to improve cycling performance in trained cyclists.[57] A reduction of oxygen cost during walking and running in physically active men has been observed with nitrate supplementation.[58] Rowing performance in well-trained rowers showed improvement after nitrate ingestion.[59] More research in needed to determine how the exergonic effects of nitrate are affected by gender, fitness status, age, dosage, and other factors.[21] Nitrate may a have cardioprotective protective effect and be involved in blood pressure regulation.[8, 30, 40]

2.3. Amino Acids Involved in Nitric Oxide Production

2.3.1. L-arginine

Arginine, 2-amino-5-guanidinovaleric acid, was discovered in 1886 by Schulze and Steiger from lupine seedlings.[60] It is a basic amino acid with a molecular weight of 174.20 g/mol and a melting point of 238˚C. One of arginine's key roles in metabolism was first elucidated with the discovery of the urea cycle.[60] Based on studies of N-balance experiments, arginine was considered nonessential in most animals and humans. Further research has proved that arginine is essential for fetal and neonatal growth, embryonic survival, as well as key for spermatogenesis and vascular tone.[15, 60-62] Scientific evidence suggests that arginine improves reproductive, immune, liver, cardiovascular, pulmonary, gastrointestinal, and renal function.[15, 60-62] Arginine also has benefits for insulin sensitivity, healing, and tissue integrity. Consequently, arginine could be used as a beneficial therapy for various conditions, including diabetes, obesity, and metabolic syndrome.[60, 62]

2.3.2. L-citrulline

Citrulline was first discovered in 1930 by Wada from watermelon juice. It is a neutral, non-protein amino acid that is ubiquitous in mammals.[63, 64] Watermelon (*Citrullus vulgaris*) is one of the few natural sources that are rich in citrulline.[27, 41] Previously, citrulline was considered an amino acid of minimal interest due to it being a non-protein amino acid. The only role it was believed to play was as an intermediate in the urea cycle.[65] Recent research demonstrated that citrulline is released by the small intestine in isolated vascularly perfused rat intestines.[66] Recent findings suggest that citrulline plays an important role in various applications and is involved in interorgan metabolism.

2.3.3. Production of nitric oxide via the L-arginine and L-citrulline cycle

Local recycling of citrulline occurs via the arginine and citrulline cycle. Arginine, used in nitric oxide production, is interconverted with citrulline.[63, 67] Arginine is converted into citrulline by the enzyme nitric oxide synthase (NOS); citrulline is converted into arginosuccinate by argininosuccinate synthetase and then converted back to arginine by argininosuccinate lyase.[67-69] Nitric oxide is produced when arginine is converted to citrulline by NOS.[68, 69] Conversion of arginine to citrulline takes place in all tissues and different types of NOS isoenzymes are expressed in various levels and in different cell types.[63, 67-69] The three isoforms of NOS are: nNOS (type-1 NOS), which is found mainly in neural cells, iNOS (type-2 NOS), which is inducible in macrophages and hepatocytes, and eNOS (type-3 NOS), which is located in endothelial cells.[67-69] Of these isoform, iNOS and nNOS are generally found in low levels.[68]

2.3.4. L-arginine and L-citrulline in sports performance and heart health

The effect of arginine and citrulline on cardiovascular health and exercise performance is a current area of research that needs further development; only a few studies have focused solely on these areas. Initial findings demonstrate that citrulline stimulated nitric oxide synthesis in healthy young adults and older adults with heart failure but did not show an increase in blood flow.[70] Another study found that citrulline supplementation did not improve exercise performance. [71] However, a different study showed supplementation with citrulline reduced blood pressure and improved endurance exercise performance.[72]

2.4. Nitric Oxide

Nitric oxide (·NO), a free radial, can act as a signaling molecule in the body, meditating various processes involved in host defense, vasoregulation, vascular homeostasis, nerve transmission, and cellular energetics; nitric oxide may also improve exercise performance. Two biological processes that produce nitric oxide are the Arginine-Citrulline-NO Cycle And The Nitrate-Nitrite-Nitric Oxide Pathway.[39, 73]

2.5. Dietary Nitrate Stability during Storage

Improperly stored raw fruits and vegetables and their products that contain high levels of nitrate may develop high levels of nitrite due to bacterial contamination. Proper storage of these fresh products is crucial to reduce bacterial conversion of nitrate to nitrite prior to human consumption. Tamme et al. studied the dynamics of nitrate and nitrite in raw vegetables during short term (48-hr) storage at ambient and refrigerated temperatures.[74] They observed that in carrot, beetroot, and radish juices, nitrate levels

significantly decreased and nitrite levels significantly increased at both temperatures; the changes were most evident at ambient temperatures.[74] Other studies have evaluated the effects of frozen storage; for example, Prasad and Chetty found that nitrate levels had a minor decrease after a one week storage period at -20ºC.[75] Further comprehensive studies are still needed to determine the stability of nitrate in different plant matrices over long term storage at different conditions.

2.6. Analysis of Nitrate and Nitrite by HPLC

Methods for analysis such as spectrophotometry, ion chromatography, gas chromatography, capillary electrophoresis, and high-performance liquid chromatography (HPLC) have been utilized to analyze nitrate and nitrite levels, [76-79] with HPLC being a common, quick and reliable method for the analysis of nitrate and nitrite from various biological matrices.[76, 77, 79, 80] Detection systems coupled with HPLC include UV/VIS absorbance and fluorescence after derivatization.[76, 77]

2.7. Analysis of Amino Acids by HPLC

Various analytical techniques, capillary electrophoresis, gas chromatography, and liquid chromatography have been used for the analysis of amino acids in a variety of samples.[81] Reverse-phase high pressure liquid chromatography (RP-HPLC) is one of the prominent techniques used in the analysis of amino acids, due to its shorter run times, simpler instrumentation, and lower costs, compared with other methods. [82-84] Few methods have been developed for the analysis of underivatized amino acids,[22, 85] due to the lack of a suitable chromophore in most amino acids. [86-88] Amino acid detection using RP-HPLC is usually carried out after pre-column derivatization; typically used reagents

are 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate (AQC), 9-fluorenylmethyl-chloroformate (FMOC-Cl), phenylisothiocyanate (PITC), dansyl-chloride, and *o*-phathalaldehyde (OPA), but many other reagents have been used. [82, 83, 86, 87, 89, 90] Each reagent has benefits and drawbacks; for example dansyl-chloride, PITC, and FMOC reagents yield stable derivatives for primary and secondary amino acids but require complex and time-consuming sample preparation. [87] OPA reagent is limited to reacting with primary amino acids and derivatives are not stable for long periods of time; however, derivatization is simple and can be carried out at 25ºC with highly fluorescent derivatives. [87, 90] Stability issues can be resolved by automated derivatization and pre-column derivatization with OPA is the most common method for amino acid analysis by HPLC. [87] Derivatized amino acids have been commonly detected by fluorescence (FLD), UV, and diode array detectors (DAD). [86, 90]

2.8. Functional Juice Blends

Functional beverages are a convenient means of delivering bioactive compounds to consumers. Juice blends may serve as an easier way to increase consumption of fruits and vegetables that contain health-promoting compounds.[7] Blending different vegetable or fruit juices together can improve the nutritional quality and acceptability of a functional juice.[91] Blending of juices can also be a means of utilizing fruits or vegetables that are high in bioactives but have bitter or off-flavors. [91, 92] Jain and Khurdiya found that blending of Indian Gooseberry (*Emblica officinalis*, also known as *Phyllanthus emblica*) in ready-to-serve beverages increased the vitamin C content and had favorable sensory qualities.[92] A juice containing litchi juice, coconut water and lemon juice was

reported to have a high sensory score and higher levels of phytochemicals.[91] An orange, kiwi, pineapple, and mango blend was found to have higher bioaccessibility of phenolic compounds and hydrophilic antioxidant activity when mixed with water versus milk or soy milk.[93] A functional juice beverage was shown to have high acceptability with a watermelon: beetroot ratio of 75:25 (v/v) and the beverage also contained higher than the recommended daily dose of vitamin C.[94] Blending of juices can lead to the development of functional beverages with targeted nutritional compositions and be a means to incorporate underutilized crops.

CHAPTER III

OPTIMIZATION AND VALIDATION OF HPLC-FLD METHOD USING OCTYLSILYL STATIONARY PHASE FOR AMINO ACIDS IN L-CITRULLINE RICH VEGETABLES

3.1 Synopsis

The proposed analytical method is based on the separation and quantification of amino acids including L-citrulline from fresh vegetables juices and commercial juices. The method separated 21 amino acids using a C_8 column stationary phase with a 5 µm particle size, 80Å pore size, 7.6% carbon load and 180 m^2/g surface area. Optimal separation conditions for amino acids analysis were obtained with 20 mM sodium acetate (solvent A) and water with organic modifier acetonitrile and methanol (solvent B; 18/50/32 V/V). The ideal pH and column temperature were found to be 5.40 and 35 ºC, respectively. LOD and LOQ values were calculated based on signal to noise ratio and were obtained in the range of as 0.02 ng/mL and 0.19 ng/mL for all investigated amino acids respectively. Relative standard deviations of peak areas for intraday analysis were within 2.7% and under 7.9% for interday analysis. The developed method was validated with six fresh vegetable juices: watermelon, cucumber, celery, calabaza squash, zucchini squash, yellow squash and commercial juice samples. Results demonstrated that L-citrulline was detected in all the fresh vegetable juices and two commercial juice samples. L-citrulline content was highest in fresh watermelon juice (719.57 ± 24.80 µg/mL) and commercial watermelon lime juice (826.48 ± 34.48 µg /mL). The optimized analytical method is rapid, sensitive, accurate and reproducible for

analysis of free amino acids including L-citrulline from different vegetable juices and other food products. To best of our knowledge, this is the first report to separate OPA amino acids derivatives using C_8 column from watermelon, cucumber, zucchini squash, yellow squash, calabaza squash, and celery in a HPLC-FLD system

3.2 Introduction

Juices have become a popular option to increase consumption of fruits and vegetables and have been associated with health benefits such as cognitive function.[9, 95-97] A variety of beneficial bioactive compounds are present in fruit and vegetable juices. Free amino acids influence the quality- aroma, taste, and color of juices and can be used to measure quality.[83, 98, 99] Amino acids are also beneficial for human health and are involved in numerous biological processes. They have functional properties including protein synthesis, cell signaling, cellular metabolism, immune response and some act as antioxidants.[15, 100] For example, L-citrulline is a non-essential amino acid engaged in interorgan metabolism.[61] It is also involved in the production of nitric oxide a signaling molecule and potent vasodilator.[65] Few fruits and vegetables are rich sources of L-citrulline. The Cucurbitaceae family, has been found to be a source of this beneficial amino acid.[101] Analysis of L-citrulline and other free amino acids in juices from these crops is important for the determination of quality and health benefits.

Various analytical techniques, capillary electrophoresis, gas chromatography, and liquid chromatography have been utilized for the analysis of amino acids in a variety of samples.[81] Reversed-phase high pressure liquid chromatography (RP-HPLC) is a commonly used technique in the analysis of amino acids, due to relatively shorter run

time, simpler instrumentation, and sample preparation steps.[82-84] Few methods have been reported for the analysis of underivatized amino acids[22, 85] and due to the lack of a suitable chromophore the sensitivity of amino acids in biological samples remains challangining.[86-88] Moreover due to acidic, basic and neutral chemical behavior of amino acids their separations are very challenging mainly due to the presence of critical pairs. Critical pairs are closely eluting amino acid derivatives such as glycine/arginine, alanine/β-alanine, and methionine/valine.[102, 103] Amino acid detection has been performed using fluorescence (FLD), UV, and diode array detector (DAD) by RP-HPLC and is usually carried out after pre-column derivatization.[86, 90] Typically used reagents for amino acid derivatization are 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate (AQC), 9-fluorenylmethyl-chloroformate (FMOC-Cl), phenylisothiocyanate (PITC), dansyl-chloride, and *o*-phathalaldehyde (OPA).[82, 83, 86, 87, 89, 90] Each reagent has an advantages and drawbacks, dansyl-chloride, PITC, and FMOC reagents yield stable derivatives for primary and secondary amino acids but have complex and time consuming sample preparations.[87] The OPA reagent is limited for primary amino acids however; the derivatization technique is simple and can be carried out at 25ºC.

Quantification of L-citrulline using HPLC is usually carried out in biological samples such as plasma, urine, and cerebrospinal fluid. [104, 105] Few methods have been reported on the analysis of L-citrulline and other amino acids from cucurbits. Amino acids quantification from watermelon by fluorescence detection using OPA-IBLC derivatives has been carried, however L-citrulline was not analyzed and the run time was over 70 minutes.[106] Dansyl chloride derivatives and HPLC-DAD have also been used for

the analysis of L-citrulline in watermelon and other cucurbits including cucumber and squash.[107, 108] The rapid determination of L-citrulline from watermelon using FMOC and HPLC-PDA has been reported, however this method was limited to only detecting L-citrulline.[22]

To the best of our knowledge no convenient analytical methods using OPA-derivatives coupled with HPLC-FLD are reported for the routine analysis of free amino acids including L-citrulline from juices of the Cucurbitaceae family. In the present study, a rapid, accurate and reproducible HPLC-FLD method using automated precolumn OPA derivatization was optimized for the analysis of free amino acids. The method was validated in the following fresh vegetable juices: watermelon (*Citrullus lanatus*), cucumber (*Cucumis sativus*), zucchini squash (*Cucurbita pepo*), yellow squash (*Cucurbita pepo*), calabaza squash (*Cucurbita pepo*), celery (*Apium graveolens*) and commercial juices.

3.3 Experimental

3.3.1 Chemicals and reagents

Amino acid standards, sodium acetate trihydrate, hydrochloric acid, 2-mercaptoethanol, HPLC grade methanol and acetonitrile were obtained from Sigma Aldrich (St. Louis, MO, USA). β-alanine and OPA were purchased from TCI Chemicals (Portland, OR, USA). L-ornithine, glacial acetic acid, and sodium borate were purchased from Thermo Fisher Scientific (Waltham, MA, USA). Nano-pure HPLC grade water (resistivity 18.2 mΩcm) was obtained from (NANO pure, Barnstead/ Thermolyne, Dubuque, IA, USA).

3.3.2 Fresh vegetable juice sample preparation

Fresh watermelon, cucumber, zucchini squash, yellow squash, and calabaza squash, and celery were obtained from a local super market (College station, TX, USA). Vegetables were rinsed thoroughly with tap water followed by nano pure water. Vegetables were finely chopped and passed through an Omega 8006 Nutrition System HD Juicer (Omega Products, Inc., Harrisburg, PA). The vegetables juices were centrifuged at 7826 ×g for 10 min and supernatants were passed though Whatman filter paper No. 1. The obtained filtrates were diluted with nano pure water and final volume was recorded. Samples were filtered through 0.40 µm cellulose syringe filter for HPLC analysis. The samples were used for optimization and validation of the analytical method.

3.3.3 Commercial juice samples

Commercial juices (J-1, J-2 and J-3) were belonging to Whole Food Market brand. Juice (J-4) belonged to BluePrint Organic and J-5 was from JUISI brand. All juices were purchased from Whole Food Market (Houston, TX, USA). Samples were kept sealed at 4 °C until analysis. Samples aliquots (1 mL) were diluted with nanopure water and centrifuged at 7826 ×g for 10 min and passed through a 0.40 µm cellulose syringe filter before HPLC analysis. Samples were store at -80 °C prior to analysis.

3.3.4 Preparation of OPA reagent

Derivatization with OPA was carried out using a slight modification of the method according to Wu and Menninger.[105] Briefly, 50 mg of *O*-phathalaldehyde were

first dissolved in 1.25 mL of HPLC grade methanol, followed by 11.1 mL of 40 mM sodium borate, 0.5 mL of 3.1% Brij-35 and 50 µL of 2-mercaptoethanol (ME).

3.3.5 HPLC-FLD conditions

Amino acids were separated using a HPLC system comprised of a Perkin Elmer Series 200 binary pump and autosampler (Perkin Elmer Life and Analytical Sciences, Shelton, Connecticut, USA). A Gastorr TG-14 inline HPLC mobile phase degasser (FLOM USA, San Diego, CA, USA) and an Eppendorf TC-50 controller with a CH-30 column heater (Eppendorf, Westbury, NY, USA). Detection was carried out by 1260 Infinity fluorescence detector controlled by an Instant Pilot model G4208A (Agilent Technologies, Santa Clara, CA, USA). The system was aligned by a PE Nelson 900 interface and a PE Nelson 600 Link box. Amino acids were separted on Zorbax Eclipse XDB-C_8 (4.6 x 150 mm, 5µm) column having 80Å pore size, 7.6% carbon load and 180 m^2/g surface area was fitted with a guard cartridge. The mobile phase consisted of solvent A) 20 mM sodium acetate buffer, and solvent B) acetonitrile/methanol/water (50/32/18). A 28 min separation of amino acid was achieved by the following gradient program, isocratic 20% B for 4 min, gradually increase from 20% to 32% B (2 min), 32% to 34% B (4 min), 34% to 38% B (3 min), linearly increase up to 95% B (9 min), kept isocratic for 5% B (2 min), return to initial condition 20% B (1 min) and remain isocratic for 4 min. The flow rate was 0.6 mL/min and the injection volume was 5µL. Prior to injection aliquots of 100 µL of juice samples and amino acid standard solution were incubated with 100 µL of OPA solution for 2 min at 25 ± 1 ºC. The derivatization procedure was automated and reaction mixture was immediately injected by HPLC-

FLD. The excitation and emission of the fluorescence detector were set at 340 nm and 455 nm respectively for monitoring the derivatized amino acids. Perkin Elmer TotalChrom version 6.3.2. software was used to process the data.

3.3.6 Method optimization

The optimization of LC method is essential for reliable and reproducible analysis of the amino acids. The peak shape, the retention times, and system pressure is greatly influenced by the pH of mobile phase and temperature, especially during amino acid analysis. Therefore, the effect of different pH and temperatures were evaluated in this study.

3.3.6.1 Effect of pH

The pH of the mobile phase plays a significant role in the amino acid separation. The effects of different buffer pH (5.40, 5.60 and 5.80) on separation and resolution of amino acids were studied. The mobile phase 20 mM sodium acetate was prepared and filtered with a solvent filtration assembly with 0.45 µm filter paper. The pH of the sodium acetate buffer (solvent A) was adjusted with 0.1N acetic acid to obtain desired pH of 5.40, 5.60, and 5.80 using a pH meter (Mettler Toledo FE20/EL20 pH meter) and used for HPLC analysis.

3.3.6.2 Column temperature

Column temperature greatly influenced the retention time (RT), selectivity and resolution of amino acids especially critical pairs. Different column temperatures (25, 30, 35, and 40 ºC) were used to studied the chromatographic separation of amino acids.

3.3.7 Validation of developed method

3.3.7.1 Calibration curve, linearity, LOD and LOQ

Stock solutions of 21 amino acids, L-aspartic acid, L-glutamic acid, L-asparagine, L-histidine, L-serine, L-glutamine, L-citrulline, L-threonine, β-alanine, L-alanine, L-tyrosine, L-tryptophan, L-valine, L-methionine, L-phenylalanine, L-isoleucine, L-leucine, L-ornithine, and L-lysine were prepared with 0.1N HCl and nanopure water (1 mg /mL) The stock solutions were further diluted to make a final concentration of 2.5 µg /mL. The final concentrations were then serially diluted 0.08, 0.16, 0.32, 0.64, 1.28 µg /mL for the calibration curve. The linearity of calibration curves of different amino acids were evaluated by injecting 5 µL of the serially diluted standard solutions. The calibration graph was obtained by plotting the HPLC peak area against their concentrations. The limit of detections (LOD) and limit of quantifications (LOQ) were determined with signal to noise ratios (S/N) of 3 and 10 respectively, by injecting the freshly prepared serial diluted standards.

3.3.7.2 Precision

The precision of the optimized method was evaluated by repeatability (intraday precision) and intermediate precision (inter-day precision) of standard amino acids mixtures and watermelon juice samples. The repeatability of the method was evaluated by the relative standard deviation (% RSD) of the individual peak area obtained from the predetermined consecutive injections (n=5) performed on each day over a period of 3 consecutive days. The % RSD of the concentration and retention time of standards and watermelon sample were determined for the all the injections.

3.3.8 Statistical Analysis

The results are expressed in means ±SE and analyzed by JMP Statistical Discovery™ (SAS) Pro.v.12.0 software package and processed by one analysis of variance (ANOVA) to evaluate significant difference ($p<0.05$) and two way analysis of variance at ($p<0.05$) was used to evaluate the effects of buffer pH and temperature on amino acid analysis. Tukey's HSD (honest significant difference) test was used for comparison of sample means. For interday and intraday analysis RSD was calculated according to the formula RSD=s/μ x 100, s is standard deviation and μ is the average.

3.4 Results and Discussion

3.4.1 HPLC method development

Chromatographic conditions were optimized to separate 21 amino acids in different vegetables using automated precolumn derivatization. Since amino acids are generally weak chromophores to conduct their separation, derivatization is an essential step. Precolumn derivatization not only intensifies amino acid detectability, but also augments the hydrophobicity of highly polar analytes which aids in reverse-phase HPLC analysis.[24]

Challenges in the analysis of amino acids included optimization of the buffer pH and column temperature for the optimal separation of amino acids. The effect of pH and column temperature was evaluated for the separation of amino acids. Various reports showed that pH plays a key role in the separation and resolution of amino acids especially for critical pairs.[23, 88]

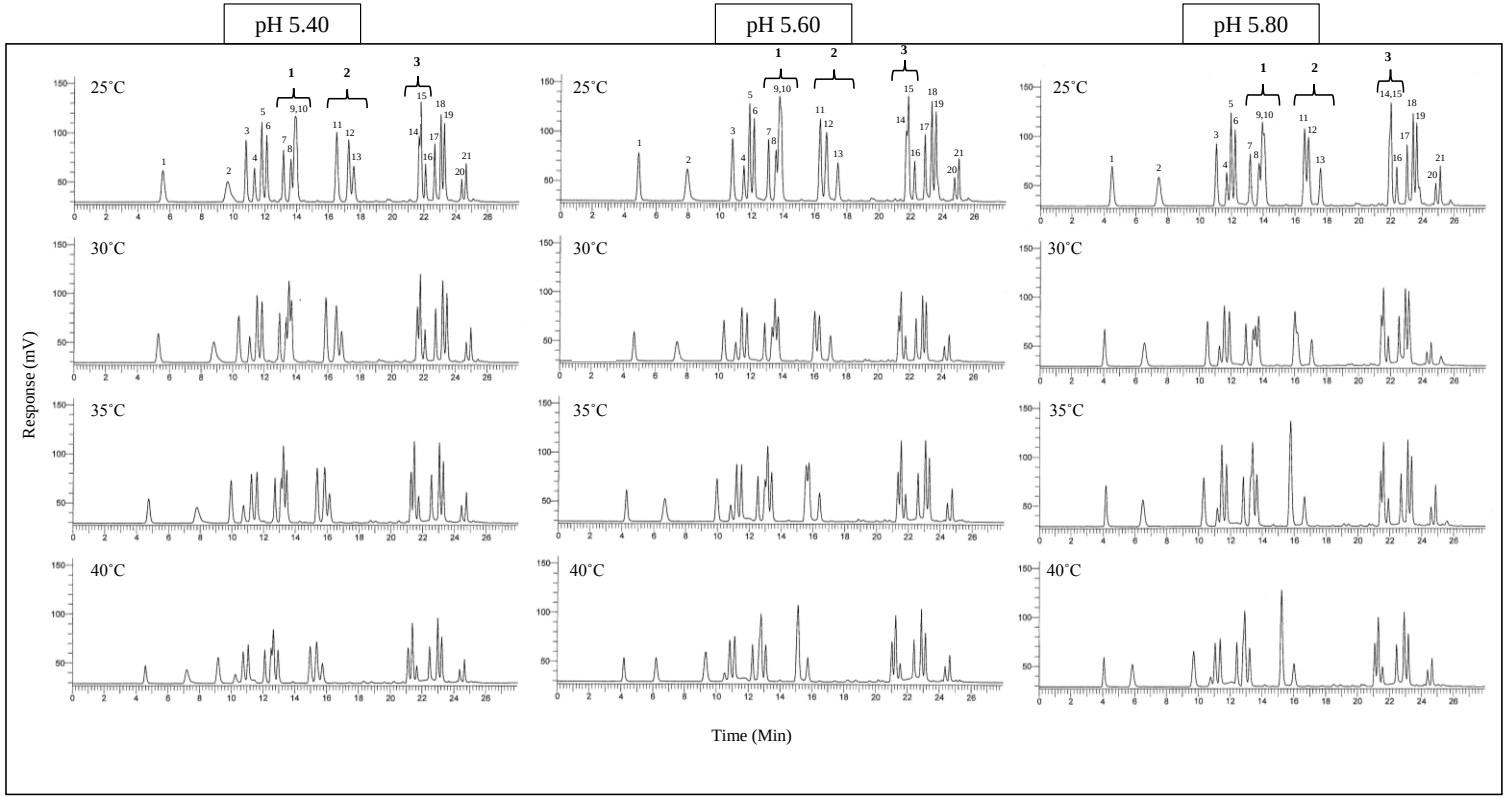

Figure 1. Chromatograms obtained from HPLC-FLD representing the effects of different temperatures (25˚C, 30˚C, 35˚C and 40˚C) at various buffer pH (A) 5.40, (B) 5.60, and (C) 5.80 on separation of 21 amino acids. The amino acid elution order is as follows: (1). L-aspartic acid, (2). L-glutamic acid, (3). L-asparagine, (4). L-histidine, (5). L-serine, (6). glutamine, (7). L-citrulline, (8). L-arginine, (9). L-glycine, (10). L-threonine, (11). L-alanine, (12).β-alanine, (13). L-tyrosine, (14). L-methionine, (15). L-valine, (16). L-tryptophan, (17). L-phenylalanine, (18). L-isoleucine, (19). L-leucine, (20). L-ornithine, and (21). L-lysine. Critical pairs shown by 1, 2 and 3.

Various reports showed that pH plays a key role in the separation and resolution of amino acids especially for critical pairs.[23, 88] To investigate the combined effect of pH and temperature, an amino acid standard mixture and watermelon juice samples were run at three pH values (5.4, 5.6 and 5.8) and four temperatures (25, 30, 35, and 40 °C). **Figure 1** displays the effects of buffer pH and temperature on amino acids separation. **Table 1** and **Table A-1** represent the effects of buffer pH and temperature on the separation and levels of amino acids present in watermelon juice and standard amino acids respectively. Results demonstrate that buffer pH and temperature greatly influence amino acid separation especially for critical pairs. In the present study, critical pair 1 consisted of arginine, glycine and threonine; critical pair 2 was alanine, β-alanine, and tyrosine, and critical pair 3 methionine and valine. For critical pair 1, the best separation was achieved at a of pH 5.40 and 35 ºC; however baseline separation of all three was not achieved. At 40 ºC baseline separation between glycine and threonine was observed with almost complete coelution of arginine and glycine. For the resolution of the second critical group, pH played a major role. The alanine and β-alanine were well separated at a pH 5.40 for all temperatures. β-alanine and tyrosine showed base line separation at pH 5.60 and 5.80, respectively. However, but with the increase in temperature, the resolution of alanine and β-alanine significantly reduced. The complete coelution of both amino acids occurred at temperature 35 ºC and 40 ºC. Finally, adequate separation of methionine and valine was achieved at a pH 5.40 and a pH 5.60. Their resolution also improved as temperature increased.

Table 1 Effect of column temperatures and different buffer pH (20 mM) on the separation and quantification of amino acids from watermelon by HPLC

	pH 5.40				pH 5.60				pH 5.80			
	25˚C	30˚C	35˚C	40˚C	25˚C	30˚C	35˚C	40˚C	25˚C	30˚C	35˚C	40˚C
Asp	74.85±1.1^{a}	59.09±1.7^{b}	46.85±0.2^{bc}	31.11±0.3^{d}	41.63±6.5^{e}	66.36±0.8^{e}	49.94±1.9^{e}	17.99±2.2^{e}	75.94±5.2^{a}	58.96±6.7^{b}	53.34±1.0^{b}	38.67±1.6^{cd}
Glu	10.29±0.2^{bc}	9.36±0.1^{c}	9.04±0.2^{c}	8.33±0.2^{c}	12.90±0.5^{d}	11.44±0.2^{d}	9.54±0.3^{d}	10.61±0.4^{d}	12.54±0.6^{a}	11.54±1.1^{ab}	10.26±0.3^{bc}	9.01±0.2^{c}
Asn	34.16±0.5^{a}	30.45±0.6^{abc}	27.22±0.0^{cd}	22.70±0.2^{d}	32.65±0.6^{e}	31.61±0.5^{e}	28.47±0.8^{e}	24.55±0.3^{e}	32.71±1.7^{ab}	28.35±2.5^{bc}	30.11±0.4^{abc}	26.62±0.9^{cd}
His	63.85±0.8^{a}	51.52±1.1^{b}	37.91±0.8^{c}	24.07±0.5^{d}	60.98±0.8^{e}	48.20±0.7^{e}	32.08±0.9^{e}	20.36±0.2^{e}	54.47±3.5^{b}	40.25±3.9^{c}	33.90±0.5c	20.13±0.8^{d}
Ser	57.09±0.6^{a}	46.97±1.0^{b}	37.19±0.3^{cd}	26.56±0.3^{e}	52.79±1.5^{f}	50.05±0.7^{f}	40.50±1.2^{f}	28.17±0.6^{f}	56.42±3.5^{a}	45.13±4.3^{bc}	41.94±0.6^{bc}	32.23±1.0^{de}
Gln	309.19±4.2^{a}	261.89±6.8^{ab}	224.54±1.8^{bc}	173.35±4.6^{d}	275.67±10.6^{e}	271.15±4.3^{e}	231.55±7.8^{e}	168.78±4.8^{e}	295.69±19.8^{a}	240.17±26.3^{bc}	243.87±2.7^{bc}	200.49±7.5^{cd}
Cit	746.90±9.8^{a}	636.09±12.5^{ab}	543.20±4.5^{bc}	366.98±37.3^{d}	752.81±9.8^{e}	648.63±10.3^{e}	552.92±16.8^{e}	469.11±12.9^{e}	715.90±49.2^{a}	571.78±62.1^{bc}	574.10±8.1^{bc}	466.82±14.5^{cd}
Arg	611.40±7.6^{a}	545.24±12.7^{abc}	498.98±1.5^{bcd}	420.64±7.8^{d}	606.00±7.8^{e}	557.47±10.7^{e}	506.72±17.3^{e}	464.04±9.9^{e}	583.29±39.3^{ab}	480.18±51.8^{abcd}	520.23±7.2^{cd}	473.53±14.7^{cd}
Thr	32.73±1.0^{a}	24.10±0.4^{b}	20.79±0.8^{bc}	17.63±0.0^{cd}	33.35±0.7^{e}	25.69±1.4^{e}	20.98±0.6^{e}	16.90±0.4^{e}	31.91±2.2^{a}	22.01±1.8^{bc}	20.36±0.2^{bc}	15.22±1.3^{d}
Ala	29.35±0.4^{a}	25.09±0.5^{ab}	21.38±0.1^{b}	16.62±0.3^{c}	29.15±0.3^{d}	25.62±0.3^{d}	21.73±0.7^{d}	PM	28.36±1.5^{a}	22.89±1.9^{b}	PM	PM
β-ala	14.41±0.2^{a}	14.06±0.3^{ab}	PM	PM	14.06±0.2^{c}	14.19±0.3^{c}	13.49±0.2^{c}	PM	15.11±0.5^{a}	12.63±0.7^{b}	PM	PM
Tyr	18.41±0.4^{a}	17.58±0.2^{a}	PM	PM	18.48±0.3^{c}	17.19±0.4^{c}	15.97±1.0^{c}	14.71±0.9^{c}	18.48±0.8^{a}	16.21±1.1^{ab}	17.23±0.1^{ab}	14.87±0.8^{b}
Met	28.03±0.4^{a}	28.45±0.5^{a}	25.17±0.5^{ab}	21.91±0.5^{b}	25.20±0.5^{c}	25.85±0.4^{c}	24.78±0.7^{c}	22.02±0.5^{c}	23.09±1.1^{b}	23.61±2.0^{b}	25.82±0.3^{ab}	23.64±0.8^{b}
Val	44.11±0.7^{a}	38.97±0.8^{ab}	36.39±0.2^{b}	39.94±0.9^{ab}	43.90±0.4^{c}	39.94±0.7^{c}	35.48±1.0^{c}	33.27±1.1^{c}	45.58±2.7^{a}	36.59±3.4^{b}	36.71±0.5^{b}	32.78±1.1^{b}
Trp	58.46±0.8^{a}	32.45±1.0^{c}	29.37±1.3^{c}	17.77±0.4^{d}	58.96±0.4^{e}	53.54±0.8^{e}	30.88±1.3^{e}	26.52±2.0^{e}	57.85±3.1^{a}	43.69±4.5^{b}	54.21±0.7^{a}	30.32±0.7^{c}
Phe	72.38±1.4^{a}	63.68±2.1^{ab}	51.58±0.4^{cd}	42.79±0.8^{d}	62.39±1.0^{e}	61.27±1.7^{e}	55.02±1.8^{e}	44.20±0.6^{e}	62.68±3.8^{ab}	57.58±5.8^{bc}	53.48±1.3^{bcd}	46.56±1.0^{d}
Iso	56.21±1.0^{a}	52.15±1.2^{ab}	48.69±0.1^{ab}	42.81±1.1^{b}	50.73±1.0^{c}	52.64±1.3^{c}	50.33±1.4^{c}	42.14±0.6^{c}	51.80±3.3^{ab}	48.11±4.7^{ab}	48.97±0.7^{ab}	45.55±1.3^{b}
Leu	39.37±0.4^{a}	33.80±0.7^{abc}	29.29±0.4^{cd}	24.31±0.9^{d}	35.99±1.5^{e}	37.15±0.7^{e}	30.12±0.6^{e}	21.76±0.6^{e}	37.59±2.2^{ab}	36.23±3.2^{ab}	32.11±0.6^{bc}	24.13±0.4^{d}
Orn	14.46±0.4^{cd}	14.11±0.5^{d}	13.42±0.4^{d}	13.76±1.1^{d}	20.69±1.5^{e}	19.31±0.6^{e}	17.92±0.5^{e}	16.88±0.9^{e}	21.73±0.9^{a}	16.19±1.3^{bcd}	17.57±0.7^{b}	17.06±0.3^{bc}
Lys	50.39±1.0^{a}	44.88±0.8^{b}	40.59±0.4^{b}	33.44±0.7^{c}	52.31±0.9^{d}	48.21±1.0^{d}	41.55±1.2^{d}	35.72±0.6^{d}	51.93±3.4^{a}	35.78±3.5^{c}	41.93±0.9^{b}	36.04±1.0^{c}

Values reported in (μg/mL), Results are mean ± standard error (n=4). PM-peak merged, Statististical differences were evaluted for the interactive effect of pH and temperature for each amino acid. Different letters represent significant differences at $P<0.05$.

A two way analysis of variance was conducted to evaluate the influence of buffer pH and column temperature on amino acids in watermelon juice matrix. All effects were statistically significant at $p<0.05$ with the majority having a significance at a level lower than 0.0001. Therefore buffer pH (5.4, 5.6, and 5.8), temperature (25 ˚C, 30 ˚C, 35 ˚C, and 40 ˚C) and their interaction significantly affected the amino acids present in watermelon juice. In the present method a pH of 5.40 and a temperature 35ºC was selected because it gave overall good resolution of all 21 amino acids. Previous studies have selected pH values of 5.60 [23] and 5.80 [88] depending on the type of derivatization and the overall system parameters. The buffer concentration can have a major impact on the HPLC system and shorten column life. For example, high concentration of buffer can cause precipitation in the system when using gradient elution.[23] Considering these issues, a 10 mM sodium acetate buffer was studied first; however inadequate peak separations were not achieved to yield reliable results (data not shown). This is likely caused by a decrease in buffering capacity at low concentrations. In accordance to previous studies[23] buffering capacity decreases with lower concentration. The optimal amino acid separations were observed with a 20 mM buffer concentration. In general, OPA in presence of thiol has been used for amino acids derivatization due to the speed, simple and lack of interference peaks during analysis.[87, 105] To alleviate issues of stability and reproducibility of the amino acids separation, the derivatization was automated using Perkin Elmer autosampler.

3.4.2 Method validation

Validation parameters including linearity, regression equation, limit of quantification (LOQ) and limit of detection (LOD) were performed for the developed method **(Table 2)**.

Table 2 Amino acid abbreviations, calibration ranges, regression equations, limits of detection (LOD) and limits of quantification (LOQ) for optimized method.

Analyte	Abbreviations	Calibration range (µg /mL)	Regression equations	R^2	LOQ (ng)	LOD (ng)
Aspartic Acid	Asp	0.08- 2.5	y=114846x-25.299	0.99	0.09	0.04
Glutamic Acid	Glu	0.08- 2.5	y=139976x-37.286	0.99	0.19	0.09
Asparagine	Asn	0.08- 2.5	y=159201x-54.391	0.98	0.09	0.04
Histidine	His	0.08- 2.5	y=66102-23.54	0.98	0.19	0.09
Serine	Ser	0.08- 2.5	y=209586x-74.714	0.99	0.04	0.02
Glutamine	Gln	0.08- 2.5	y=164803x-48.781	0.99	0.04	0.02
Citrulline	Cit	0.08- 2.5	y=148727x-56.682	0.99	0.04	0.02
Arginine	Arg	0.08- 2.5	y=136943x-39.846	0.99	0.09	0.04
Glycine	Gly	0.08- 2.5	y=210538x-80.784	0.98	0.09	0.04
Threonine	Thr	0.08- 2.5	y=176229x-51.647	0.99	0.09	0.04
Alanine	Ala	0.08- 2.5	y=227750x-71.214	0.99	0.04	0.02
β-alanine	β-ala	0.08- 2.5	y=185265x-86.46	0.98	0.19	0.09
Tyrosine	Tyr	0.08- 2.5	y=127333x-49.682	0.99	0.09	0.04
Methionine	Met	0.08- 2.5	y=153857-50.886	0.99	0.19	0.09
Valine	Val	0.08- 2.5	y=235227x-90.799	0.99	0.04	0.02
Tryptophan	Trp	0.08- 2.5	y=89378x-28.756	0.99	0.19	0.09
Phenylalanine	Phe	0.08- 2.5	y=120668-39.179	0.99	0.19	0.09
Isoleucine	Iso	0.08- 2.5	y=173491x-58.336	0.99	0.19	0.09
Leucine	Leu	0.08- 2.5	y=168689x-54.055	0.99	0.09	0.04
Ornithine	Orn	0.08- 2.5	y=28857x-14.858	0.98	0.39	0.19
Lysine	Lys	0.08- 2.5	y=78059x-34.164	0.99	0.39	0.19

x = concentration of their respective compounds.
y = peak area (AU)

Excellent linearity was observed in all 21 amino acid standards by plotting the concentration as a function of peak area obtained from HPLC-FLD analysis. The

correlation coefficient was found to be >0.98. The LOQ and LOD were observed by the signal to noise (S/N) ratio and their low values confirmed the good sensitivity of the developed method. Intraday and interday precision were evaluated for 3 days using standard amino acids and watermelon sample. The results of inter- and intraday variation for concentration and retention times for standards and watermelon are presented in **Table A-2**. The intraday and interday means for standard mixture and watermelon juice are compiled in **Table 3.**

Table 3 Intraday and interday analysis of amino acids (µg /mL) by HPLC-FLD

Analyte	Mean Concentration		% Recovery		Mean Retention Time			
	Watermelon		Standard		Watermelon		Standard	
	Intra-day	Inter-day	Intra-day	Inter-day	Intra-day	Inter-day	Intra-day	Inter-day
Asp	51.79 ±3.45	54.17 ±4.54	116.37	113.99	5.51 ±0.03	5.42 ±0.19	5.47 ±0.07	5.34 ±0.21
Glu	9.85 ±0.25	9.45 ±0.57	110.51	107.75	9.09 ±0.10	8.91 ±0.38	8.96 ±0.08	8.99 ±0.23
Asn	29.42 ±1.25	29.07 ±2.04	121.44	117.55	10.50 ±0.04	10.39 ±0.27	10.46 ±0.09	10.41 ±0.12
His	49.51 ±2.20	47.98 ±3.55	127.23	123.16	11.19 ±0.05	11.10 ±0.25	11.23 ±0.08	11.15 ±0.10
Ser	45.50 ±2.16	44.63 ±3.23	95.91	96.65	11.59 ±0.04	11.50 ±0.23	11.64 ±0.06	11.55 ±0.10
Gln	257.02 ±14.05	254.10 ±19.41	103.13	104.53	11.87 ±0.03	11.80 ±0.22	11.94 ±0.04	11.86 ±0.09
Cit	646.77 ±18.92	625.32 ±49.35	96.41	95.59	12.91 ±0.05	12.80 ±0.25	12.93 ±0.04	12.88 ±0.06
Arg	518.73 ±38.01	533.75 ±20.53	88.87	85.66	13.41 ±0.04	13.28 ±0.27	13.39 ±0.04	13.37 ±0.06
Gly	ND	ND	127.46	125.02	ND	ND	13.56 ±0.03	13.52 ±0.07
Thr	22.10 ±1.31	22.54 ±1.80	107.58	106.27	13.75 ±0.04	13.62 ±0.29	13.76 ±0.04	13.71 ±0.07
Ala	23.49 ±1.62	23.85 ±1.70	112.58	108.18	16.05 ±0.05	15.88 ±0.40	16.05 ±0.05	16.01 ±0.08
β-ala	12.41 ±0.22	12.59 ±0.50	124.27	123.79	16.55 ±0.07	16.38 ±0.45	16.70 ±0.07	16.65 ±0.09
Tyr	16.36 ±0.65	15.95 ±0.94	93.71	94.41	16.66 ±0.05	16.48 ±0.44	17.06 ±0.11	17.03 ±0.10
Met	24.95 ±1.45	25.14 ±1.90	96.96	92.94	21.56 ±0.04	21.48 ±0.29	21.60 ±0.05	21.55 ±0.17
Val	37.53 ±1.32	36.74 ±2.64	108.76	105.15	21.74 ±0.04	21.66 ±0.31	21.78 ±0.04	21.73 ±0.20
Trp	24.99 ±1.29	25.58 ±2.63	83.79	81.39	22.01 ±0.03	21.91 ±0.33	22.09 ±0.03	22.01 ±0.18
Phe	68.91 ±3.66	66.66 ±5.24	111.98	109.26	22.75 ±0.05	22.62 ±0.45	22.79 ±0.04	22.68 ±0.19
Iso	56.85 ±3.20	54.25 ±4.38	127.00	123.43	23.22 ±0.04	23.05 ±0.51	23.23 ±0.03	23.13 ±0.20
Leu	39.26 ±1.70	37.32 ±2.83	117.68	116.42	23.46 ±0.04	23.28 ±0.55	23.43 ±0.07	23.36 ±0.20
Orn	15.35 ±0.52	15.04 ±0.72	125.61	123.27	24.60 ±0.02	24.30 ±0.77	24.63 ±0.03	24.50 ±0.21
Lys	38.64 ±2.11	39.38 ±3.03	94.60	92.55	24.89 ±0.02	24.65 ±0.78	24.93 ±0.03	24.78 ±0.21

For precision analysis 5 injections (intraday) were performed within a day for 3 consecutive days (interday). Results are represented in mean±standard deviation and % recovery for amino acid standard mixture. % Recovery= (amount recovered/actual amount injected)*100. ND not detected.

Results demonstrated that for amino acid standards the % RSD for RT was below 1.3% (n=5) for intraday and within 4% for interday analysis (n=5). For watermelon juice, the intraday % RSD for RT was lower than 1.1% and for interday analysis was found to be within 3.5 %. The %RSD for the standards concentration was under 2.5% for intraday and lower than 8% for interday analysis. For watermelon concentration the %RSD was below 7% and 9.5% for intraday and interday analysis respectively.

3.4.3 Amino acid quantification of fresh vegetable juices

The optimized HPLC-FLD method was used to determine the amino acid profile of six different fresh vegetable juices. **Figure 2** depicts the comparative amino acids chromatograms of the standard mixture, watermelon, cucumber, celery, zucchini squash, yellow squash, and calabaza squash juices. The amino acid content present in various vegetable juices are presented in **Table 4**. Results demonstrated that L-citrulline (604.59 ±20.92 µg/g) was predominant in watermelon juice followed by arginine (523.82± 18.13 µg/g). The lowest was glutamic acid (9.21±0.19 µg/g) and glycine was not detected in the present sample. Our results are consistent with others findings that report high levels of citrulline in watermelon.[41]

Published L-citrulline levels in watermelon vary significantly depending on variety and location with the reported range being 0.5 to 3.6 mg g^{-1} of fresh sample.[45, 109] The lower levels of glycine in watermelon samples were also reported.[107, 110] Cucumber juice was rich in glutamine (445.13±18.44µg/g) but had low tyrosine (11.05±0.33 µg/g). Glutamine has been previously reported as the main amino acid in cucumber.[107]

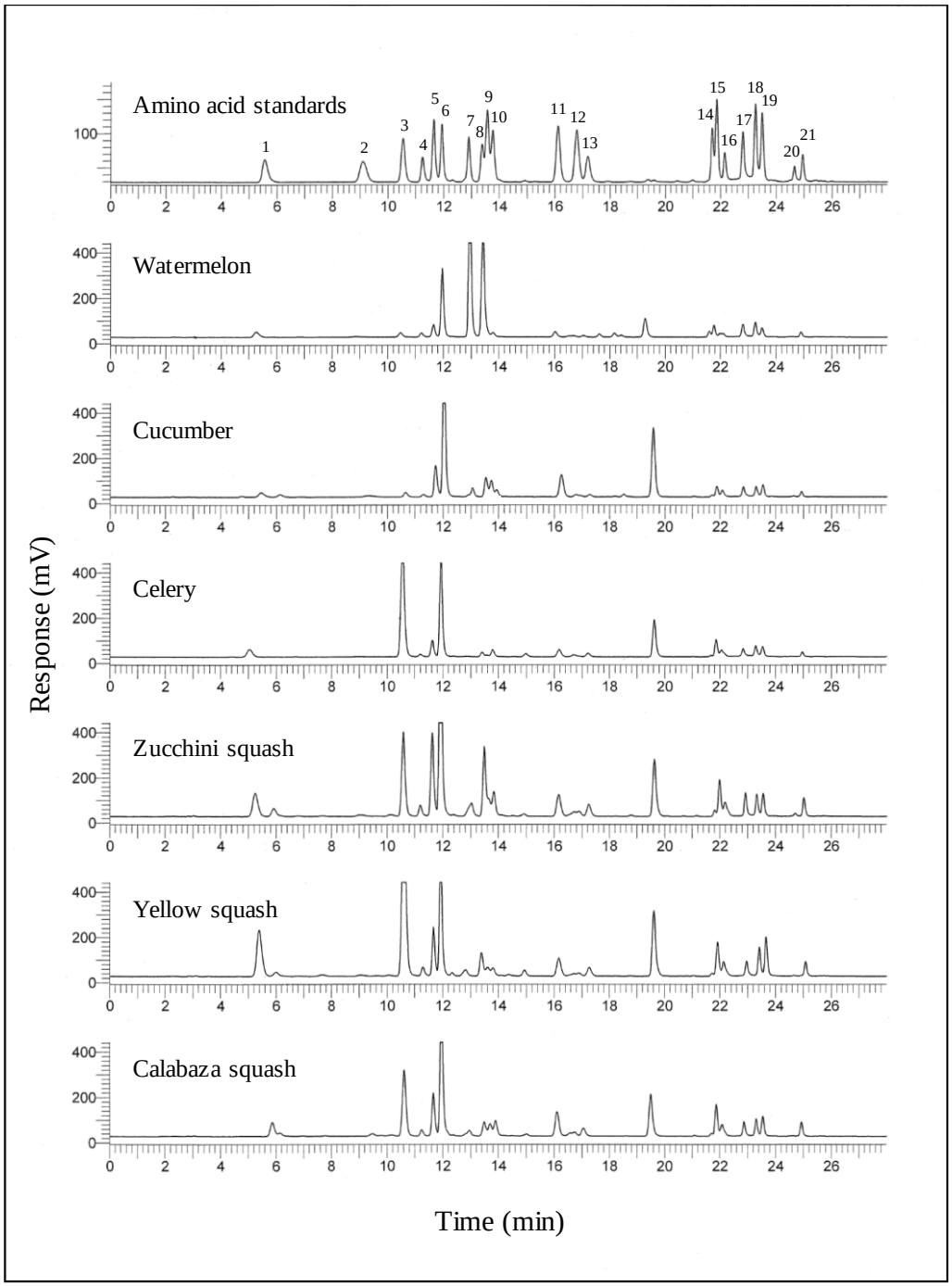

Figure 2 Comparative chromatograms of amino acid standard mixture, watermelon, cucumber, celery, zucchini squash, yellow squash, and calabaza squash juices. The amino acid elution order is as follows: (1). L-aspartic acid, (2). L-glutamic acid, (3). L-asparagine, (4). L-histidine, (5). L-serine, (6). glutamine, (7). L-citrulline, (8). L-arginine, (9). L-glycine, (10). L-threonine, (11). L-alanine, (12).β-alanine, (13). L-tyrosine, (14). L-methionine, (15). L-valine, (16). L-tryptophan, (17). L-phenylalanine, (18). L-isoleucine, (19). L-leucine, (20). L-ornithine, and (21). L-lysine.

Table 4 Amino acid content in various fresh vegetable juices analyzed by HPLC-FLD

Analyte	Watermelon	Cucumber	Celery	Z. Squash	Y. Squash	C. Squash
Asp	54.15±1.68^{de}	36.86±1.19^{de}	65.71±1.22^{c}	103.76±3.19^{de}	223.33±17.57^{c}	62.92±7.99^{cde}
Glu	9.21±0.19^{h}	12.89±0.75^{e}	ND	12.70±1.65^{i}	6.46±0.57^{j}	13.52±0.84^{gh}
Asn	28.48±0.80^{efgh}	22.51±0.51^{e}	486.86±11.36^{a}	257.97±19.96^{b}	465.34±28.59^{a}	274.14±18.26^{b}
His	46.80±1.59^{def}	26.48±1.64^{de}	26.95±1.04^{fghi}	61.56±3.27^{fg}	51.23±6.02^{defgh}	45.76±3.93^{defgh}
Ser	43.79±1.40^{defg}	86.91±5.08^{bc}	50.28±0.83^{cde}	129.20±4.59^{d}	75.96±6.04^{de}	96.49±7.96^{c}
Gln	248.22±8.32^{c}	445.13±18.44^{a}	299.75±15.16^{b}	441.66±9.05^{a}	308.41±39.35^{de}	395.34±27.44^{a}
Cit	604.59±20.92^{a}	42.02±2.88^{de}	8.89±1.63^{i}	48.79±3.19^{fghi}	19.17±1.36^{b}	27.58±2.98^{efgh}
Arg	523.82±18.13^{b}	81.66±8.12^{bc}	23.56±0.32^{ghi}	195.64±9.99^{c}	81.31±11.92^{ghij}	47.88±7.14^{defg}
Gly	ND	56.75±6.39^{cd}	9.63±0.18^{i}	26.01±1.27^{ghi}	18.92±1.62^{d}	23.17±2.11^{fgh}
Thr	22.40±0.68^{efgh}	28.67±1.95^{de}	29.24±0.49^{fgh}	49.35±1.75^{fghi}	20.34±1.17^{ghij}	43.99±4.32^{defgh}
Ala	23.71±0.68^{efgh}	110.69±18.75^{b}	29.19±0.46^{fgh}	49.86±3.07^{fghi}	40.49±2.62^{efghij}	67.76±2.23^{cd}
β-ala	12.61±0.15^{gh}	15.59±0.40^{e}	14.28±0.47^{hi}	16.64±0.25^{i}	13.34±1.25^{hij}	14.12±1.02^{gh}
Tyr	15.63±0.38^{fgh}	11.05±0.33^{e}	9.35±0.20^{i}	19.66±1.19^{hi}	15.27±1.27^{hij}	20.24±1.24^{fgh}
Met	25.32±0.78^{efgh}	11.62±0.88^{e}	12.15±3.13^{hi}	13.37±1.07^{i}	6.54±0.66^{j}	7.49±0.76^{h}
Val	37.25±1.62^{defgh}	23.99±2.26^{de}	42.34±1.10^{def}	50.14±3.52^{fghi}	46.02±4.62^{defghi}	55.64±4.41^{def}
Trp	25.46±0.69^{efgh}	43.21±2.10^{de}	55.88±0.71^{cd}	58.33±2.63^{fgh}	54.45±7.65^{defg}	55.55±3.58^{def}
Phe	65.11±2.26^{d}	41.57±1.66^{de}	35.68±0.40^{efg}	49.86±3.01^{fghi}	33.68±2.88^{fghij}	46.17±4.49^{defgh}
Iso	53.01±1.90^{de}	32.57±2.10^{de}	34.73±0.66^{efg}	35.97±2.91^{fghi}	44.35±4.09^{defghij}	40.81±3.82^{defgh}
Leu	36.32±1.23^{defgh}	35.67±1.22^{de}	34.60±0.93^{efg}	40.20±3.33^{fghi}	60.26±5.99^{def}	43.71±4.91^{defgh}
Orn	14.93±0.28^{fgh}	28.35±4.99^{de}	11.04±0.17^{hi}	35.56±3.24^{fghi}	8.56±0.81^{ij}	8.76±0.43^{gh}
Lys	38.23±1.11^{defgh}	31.47±1.14^{de}	33.30±0.62^{efg}	72.51±5.39^{ef}	49.79±4.69^{defgh}	64.00±5.57^{cde}

Data presented is mean (μg/g fresh weight) ± standard error. ND, not detected. Statistical differences evaluted within each vegetables amoung amino acids. Different letters represent significant differences at $p<0.05$.

Aspartic acid (486.86±11.36 µg/g) was the highest amino acid in celery juice but glutamic acid was not detected. The level of glutamine (441.66±9.05 µg/g) in zucchini squash was high. Yellow squash had high levels of asparagine (465.34±28.59 µg/g) while lower levels of glutamic acid (6.46±0.57 µg/g). Calabaza squash juice contained higher levels of glutamine (395.34±27.44 µg/g) and low levels of methionine (7.49±0.76 µg/g). The total amino acids were highest in watermelon (1929.03±63.54 µg/g) followed by zucchini squash juice (1769.75±63.88 µg/g), yellow squash (1643.22±145.25 µg/g), calabaza squash (1455.05±101.21 µg/g), and celery (1313.44±33.96 µg/g). Cucumber juice contained the lowest level of amino acids (1226.67±76.64 µg/g).

3.4.4 L-Citrulline content in fresh vegetable juices

L-citrulline is a non-proteogenic and nonessential amino acid that is ubiquitous in mammals, plants, fungi and bacteria. [101] It was first discovered in 1930 in watermelon juice, since then it has been researched for its clinical and therapeutic uses.[64, 65, 101] It plays various roles in metabolism including the regulation of nitric oxide which affects cardiovascular health and also acts as an antioxidant.[65, 111] All vegetables in the present study are a source of L-citrulline, which ranged from 8.89±1.63 µg/g to 604.59±20.92 µg/g. Watermelon juice had the highest level of L-citrulline (604.59±20.92 µg/g) followed by zucchini squash (48.79±3.19 µg/g), calabaza squash (27.58±2.98 µg/g), cucumber (42.02±2.88 µg/g), yellow squash (19.17±1.36 µg/g), and celery juice (8.89±1.63 µg/g). The *Cucurbitaceae* family is known for accumulating L-citrulline, specially watermelon which has been found to have very high levels.[101] Interestingly, in

contrast to a previous report [107] our finding show the presence of L-citrulline in yellow squash.

3.4.5 Application of developed method for commercial juice analysis

The developed method was further validated by analysis of commercially available fresh pressed juices (J-1 to J-5). Results of amino acids in commercial juices are presented in **Table 5**.

Table 5 Content of amino acids present in five commercial juices.

Amino acids	Commercial Juice Samples (μg/mL)				
	J-1	J-2	J-3	J-4	J-5
Asp	140.83±3.3^{bc}	137.67±5.6^{cd}	18.52±0.6^{fg}	70.74±4.25^{c}	66.88±8.4^{bc}
Glu	13.10±3.4^{c}	15.01±1.3^{f}	36.45±1.3^{de}	130.56±8.07^{b}	51.25±6.0^{bc}
Asn	335.27±34.0^{b}	293.22±12.0^{b}	107.45±3.6^{a}	245.20±10.00^{a}	71.55±3.5^{bc}
His	49.54±7.9^{c}	43.81±0.7^{ef}	32.39±1.0^{de}	21.41±0.21^{d}	48.97±1.0^{bc}
Ser	ND	ND	51.78±2.0^{c}	61.20±5.69^{c}	ND
Gln	ND	ND	76.33±2.3^{b}	147.08±1.10^{b}	ND
Cit	ND	ND	17.94±0.1^{fg}	ND	826.48±34.5^{a}
Thr	131.51±13.3^{bc}	111.74±5.3^{cde}	22.67±0.7^{f}	33.69±1.82^{d}	32.72±2.3^{c}
Ala	210.12±26.3^{bc}	187.57±7.7^{c}	17.34±0.7^{fg}	21.55±0.84^{d}	72.04±3.8^{bc}
β-ala	ND	ND	40.22±1.9^{d}	16.20±0.01^{d}	24.06±0.1^{c}
Tyr	825.26±42.0^{a}	677.17±49.3^{a}	12.18±1.1^{gh}	ND	97.66±5.0^{b}
Trp	30.35±4.0^{c}	28.04±1.1^{ef}	9.04±0.2^{h}	12.87±0.71^{d}	27.76±1.1^{c}
Iso	80.77±8.7^{c}	72.48±2.2^{def}	8.37±0.3^{h}	15.92±0.86^{d}	38.80±3.0^{c}
Leu	76.28±6.8^{c}	68.46±1.6^{def}	21.40±0.4^{f}	18.71±0.20^{d}	23.97±0.5^{c}
Lys	108.93±15.9^{bc}	66.22±2.7^{def}	31.77±0.6^{e}	19.46±2.17^{d}	37.91±1.4^{c}

(J-1) Juice 1: ginger, beetroot, and carrot. (J-2) Juice 2: carrot and beetroot. (J-3) Juice 3: kale and pineapple. (J-4) Juice 4: kale, apple, and lemon. (J-5) Juice 5: Watermelon and lime. ND, not detected. Statististical differences evaluted within each juice amoung amino acids. Different letters represent significant differences at $p<0.05$. Data presented is mean±standard error. ND, not detected.

Results show that commercial juices had lower amino acid content than fresh juices except for Juice 1 (J-1) and Juice 2 (J-2). For overall amino acid content J-1 had the highest amount (2001.95 µg/mL) followed by J-2 (1701.38 µg/mL), J-5 (1395.98 µg/mL), J-4 (814.58 µg/mL), and J-3 (503.84 µg/mL). J-1, a freshly squeezed juice, comprised of ginger, beetroot, and carrot had tyrosine (825.26±41.98 µg/mL) as the highest amino acid. The lowest amino acid was glutamic acid (13.10±3.41 µg/mL). The same trend was observed with J-2, a freshly squeezed juice of carrot and beetroot. It had 677.17±49.32 µg/mL of tyrosine and 15.01±1.25 µg/mL of glutamic acid. J-3, made up of freshly squeezed juices of kale and pineapple, had 107.45±6.61 µg/mL of asparagine and 8.37±0.34 µg/mL of isoleucine (lowest level). Other amino acids were below the limit of detection. J-4 (cold-pressed juice) contained kale, apple and lemon had an asparagine level of 245.20±10.00 µg/mL and 12.87±0.71 µg/mL tryptophan (lowest). J-5 was a raw cold pressed watermelon-lime mixture. Interestingly, serine, glutamine, arginine, methionine, valine, phenylalanine, and ornithine were not detected this commercial juice in contrast to fresh watermelon juice. Conversely, L-citrulline was found in higher concentration (826.48±34.48 µg/mL) in J-5 than fresh watermelon juice (719.57±24.80 µg/mL µg/mL) used in our study. This may be due to the cultivar used for the commercial juice and their growing conditions.

3.5 Conclusions

The optimized analytical method using C_8 stationary phase separated 21 amino acids in 28 minutes for the first time. The optimized method has high sensitivity and reproducibility. The method was tested with different fresh and commercially available

vegetable juices. L-Citrulline, a valuable amino acid for health promotion, was found to have reliable retention times and was detected in all fresh juices. This method has a high potential to be used for routine amino acid analysis in various food matrixes and vegetables.

CHAPTER IV

STORAGE STABILITY OF DIETARY NITRATE AND PHENOLIC COMPOUNDS IN BEETROOT (*BETA VULGARIS*) AND ARUGULA (*ERUCA SATIVA*) JUICES

4.1 Synopsis

Nitrate and polyphenols from the diet may enhance the production and bioavailability of nitric oxide, a radical signaling molecule critical for cardiovascular health. Beetroot and arugula are rich sources of dietary nitrate and polyphenols. The storage stability of these bioactives is of importance for their functions. In the present study, the stability of nitrate and total phenolics in beetroot and arugula juices was measured over one month at different temperatures (25, 4, -20 and -80˚C). Beetroots and arugula were juiced using an Omega HD juicer and then stored at various temperatures for different periods. The levels of nitrate were measured by reversed phase HPLC using a C_{18} column. The initial levels of nitrate were 4965.34 ± 72.69 and 6310.20 ± 24.79 µg/mL for beetroot and arugula, respectively. At 25˚C, nitrate degradation initiated within 24 h, whereas at 4˚C nitrate degradation initiated after 4 days. At -20˚C and -80˚C, nitrate levels remained stable for one month. Assays for total phenolics and free radical scavenging activity demonstrated that phenolics content and antioxidant activity varied with the storage conditions. For beetroot at 25˚C and 4˚C, a decrease in total phenolics and antioxidant activity was observed, while at -20˚C and -80˚C levels remained relatively stable. However, for arugula juice at 25˚C and 4˚C, an increase in total phenolics content and antioxidant activity was observed after one month. UPHLC-HR-QTOF-MS analysis demonstrated that during storage, flavonoid glucosides were

broken down to their aglycone moieties and lower phenolics, thus resulting in higher total phenolics and antioxidant activity. In conclusion, beetroot and arugula juices required suitable temperatures to prevent the degradation of nitrate and maintain the nutritional value.

4.2 Introduction

Nitric oxide (NO) is a ubiquitous gasotransmitter involved in various pathological and physiological processes in the body.[101, 112] Blood flow, platelet aggregation, and vascular tone are all modulated via NO, hence its impairment in the vascular system is associated with cardiovascular diseases.[101] Due to its importance, approaches to enhance NO production and bioavailability for its use as a therapeutic agent have been a prominent area of research.[101, 111, 113] Dietary nitrate and polyphenolic compounds present in fruits and vegetables are involved in supplementing and enhancing NO-producing pathways.[8, 40, 112, 114]

It has been hypothesized that the cardioprotective benefits of a Mediterranean diet may in part be attributed to the presences of polyphenols and nitrates from the plant foods consumed.[8, 115] Vegetables contribute approximately 85% of nitrate present in the diet.[8] For biological activity, dietary nitrate must be secreted in saliva and reduced to nitrite,[8, 56, 116, 117] by facultative anaerobic bacteria in the oral cavity.[56, 118] Nitrite is further reduced to NO after swallowing in the acidic conditions of the stomach and in other parts of the body.[118] Dietary polyphenols act as antioxidants that scavenge free radicals that may cause damage.[115] Epidemiological studies suggest that with consumption of flavonoids there is an inverse association with cardiovascular disease.[114]

The main thought is that flavonoids influence NO by protecting the bioactivity of NO derived in the endothelium.[112] The flavonoid luteolin was found to possibly affect cardiovascular function by stimulating NO-dependent vascular dilation in rat aortic rings.[119]

Beetroot and arugula are considered rich sources of polyphenols and dietary nitrate. Beetroot contains high levels of nitrate with a range of (644–1800 mg/kg) reported in the literature.[8] Beetroot further contains beneficial phenolic compounds such as coumaric acid and ferulic acid, as well as betalains and betacyanins, which are responsible for the red pigmentation in the plant.[120-122] Arugula has the highest reported levels of nitrate, ranging from 1213–2650 mg/kg.[8] The main polyphenols in arugula are flavonoids with quercetin, kaempferol, and isorhamnetin being predominant.[32] Beetroot and arugula consumed fresh or in food products such as juices may be considered functional foods, due to their polyphenols and nitrate content, which may act as therapeutic agents due to their potential to enhance NO production and bioavailability.

The stability of nitrate and polyphenolic compounds present in beetroot and arugula and their food products is important when considering their functional properties. During storage, nitrate can be reduced to nitrite due to bacterial nitrate reductases.[8] In freshly prepared home vegetable juices, nitrite content is negligible; however, after 2 days of room temperature storage, increases to almost 600 mg/L can occur, but storage at 4˚C can prevent this conversion.[74] Several reports have studied the stability of betalains and betacyanins in beetroot and other crops.[120, 123-126] A principal sign of the degradation betalains and betacyanins is a loss of color due to their pigment

properties.[126] The stability of flavonoids in different plant matrices has also been researched extensively.[110, 127-129] It has been observed that a 1-month storage period of apple juice at refrigerator and room temperatures did not lower the concentration of total quercetin glycosides, total catechins, chlorogenic acid, phloridzin, or cyanidin galactoside.[130]

Vegetables juices from beetroot and arugula may have the potential to enhance nitric oxide production due to their phenolic compounds and nitrate content.[114] To determine the stability of these beneficial compounds in the juices during storage, nitrate, total polyphenolics and their radical scavenging activity were evaluated for 32 days at different temperatures. The bioactives present in beetroot and arugula juice at 0 and 32 days were identified by ultrahigh performance liquid chromatography with high-resolution quadrupole time of flight mass spectral (UPLC-HR-QTOF-MS) analysis to confirm their stability during storage. To the best of our knowledge, this is the first report to demonstrate nitrate and phenolic compounds in beetroot and arugula during 32 days of storage, and to test radical scavenging activity

4.3 Materials and Methods

4.3.1 Standards and chemicals

Nitrate and nitrite standards were obtained from Alfa Aesar (Ward Hill, MA). L-ascorbic acid, gallic acid, sodium carbonate, 2,2-diphenyl-1-picrylhydrazyl, Folin-Ciocalteu (FC) reagent, methanol, HPLC grade acetonitrile and phosphoric acid were obtained from Sigma-Aldrich (St. Louis, MO). Nano-pure water was used for analysis

and sample preparation obtained from NANO pure Barnstead/ Thermolyne (Dubuque, IA).

4.3.2 Juicing of plant materials

Fresh beetroot (*Beta vulgaris*) and organic baby arugula (*Eruca sativa*) were purchased at a local grocery store (College Station, TX, USA). The beetroot and arugula samples were rinsed thoroughly with tap water followed by nano pure water then inedible parts were removed. For beetroot, the bulbs were cut into small pieces before juicing. The beetroot pieces and arugula leaves were then juiced separately using an Omega 8006 Nutrition System HD Juicer (Omega, Harrisburg, PA, USA).

4.3.3 Sample storage conditions

Both the juice samples were stored at four different temperatures: 25, 4, -20, and -80°C, for 32 days in triplicates. Samples from various temperatures were taken for phytochemical analysis periodically (0, 2, 4, 8, 16, and 32 days). Initial extraction and analysis after juicing was considered the initial day (day 0). For nitrate and nitrite analysis, two additional time points at 8 h and 1 day were also evaluated to understand the degradation pattern.

4.3.4 Analysis of nitrate and nitrite

All glassware and equipment during processing was rinsed with nano-pure water to reduce nitrate contamination from the environment. The beetroot and arugula juice from each time point was taken and allowed to come to 25°C then centrifuged at 800×g for 5 min. (Beckman Model TJ-6, Beckman Instruments, Inc., Palo Alto, CA) and the supernatant decanted and passed through a 0.45-µm cellulose filter. The filtered samples

were diluted with nano-pure water 1:20 ratio. Initial (day 0) samples were extracted immediately following juicing then analyzed by high pressure liquid chromatography (HPLC). The rest of the juices were stored at different temperatures over different time points and extracted at the predetermined time points and analyzed by HPLC. The HPLC system consisted of a Waters 1525 binary pump, auto injector (20 µL loop), a 2996 photodiode array detector and "Empower" software program. A C_{18} Gemini column (250mm × 4.6 mm) (Phenomenex, Torrance, CA) having particle size 3 µm, pore size 110 Å, and carbon load 14% was used for nitrate and nitrite determination using an isocratic mobile phase of 0.3 M phosphoric acid: ACN (9:1) at a flow rate of 0.6 ml/min. Chromatograms were acquired at 210 nm and peak assignments were made based on respective standards. The calibration curves of nitrate and nitrite were prepared in nano pure water. Stock solutions of 1000 µg/ml were prepared and serially diluted to 0.5 µg/ml to prepare the calibration curve, for the calculation of nitrate and nitrite concentration present in the samples.

4.3.5 Methanol Extraction

All samples from different storage periods were used for total phenolics and free radical scavenging activity. Juice samples (0.5 mL) were extracted with methanol 4.5 mL by vortexing for 2 min and sonication for 30 min. The samples were centrifuged 800 ×g for 5 min, passed through a 0.45-µm PTFE syringe filter, and stored at -20°C until further analysis.

4.3.6 Analysis of phenolic compounds using UPLC-HR-ESI-QTOF-MS

To understand the stability of health-promoting compounds from beetroot and arugula during storage, at 0 day and 32 days (25°C) and 32 days (-80°C) samples were analyzed by UHPLC-ESI-HR-QTOFMS. The analysis was performed on a 1290 Agilent LC system (Agilent, Santa Clara, CA) attached to a maXis impact mass spectrometer (Bruker Daltonics, Billerica, MA). The phenolic compounds were separated on a Zorbax eclipse plus C_{18} column (1.8 μm, 50 × 2.1 mm) (Agilent, Santa Clara, USA) with a flow rate of 0.2 mL/min at 65 °C. A mobile phase consisting of (A) 0.1% formic acid in water and (B) 0.1% formic acid in water: acetonitrile (3:7). Elution of phenolics was performed by using gradient programming as follows: 3 min isocratic A (100%), followed by 0 to 100% B (3-16 min), 3 min isocratic B (100%), then 100% to 0 (1 min). The injection volume was 2 μL and post-run equilibrium time was 2 min.

Mass spectral analysis was performed by electrospray ionization (ESI) in positive ionization mode. MS and bbCID data were acquired at *m/z* range of 25–2000 amu. The ion source capillary voltage was 4,200 V and nebulizer gas pressure was 2.8 bar. The flow rate and temperature of drying gas were 8.0 L/min and 220 °C, respectively. Nitrogen was used for both nebulizer and drying gas. The transfer time of the source was 72 μs and the prepulse storage time was 1 μs. The quadrupole MS and bbCID collision energy were set at 5 and 70 eV, respectively. Sodium formate solution (1 mM sodium hydroxide and water: isopropanol (1:1) with 0.2% formic acid) was used for the instrumentation calibration. Internal mass scale calibration of individual samples was

performed by injecting the above calibrant at the end of each run using a Cole Palmer syringe pump (Vernon Hills, Illinois, USA).

4.3.7 Total phenolics measurement

The Folin-Ciocalteu (FC) method [131] was used to determine the total phenolics from beetroot and arugula samples. In a 96-well plate, 20 µL of methanolic extract was adjusted to 180 µL with nano pure water. Subsequently, 40 µL of 25% solution FC reagent was added and incubated for 10 min at room temperature. This was followed by the addition of 50 µL of sodium carbonate and incubation at room temperature for 20 min. The absorbance of blue color was measured at 760 nm using a KC-4 Microplate Reader (BioTek Instruments, Winooski, VT, USA). The results were expressed as gallic acid equivalents.

4.3.8 2,2-Diphenyl-1-picrylhydrazyl (DPPH) assay

DPPH radical-scavenging activity was measured using the method described by Jayaprakasha et al., [132] with slight modifications. Methanol extracts (20 µL) were pipetted into 96-well micro plates and the total volume of each well was adjusted to 100 µL with MeOH. Then 0.1 mM methanolic DPPH solution (180 µL) was added to sample wells and samples were incubated for 20 min in the dark. The absorbance was measured at 515 nm using a KC-4 Microplate Reader. Since the samples contained color, blank wells were prepared by pipetting samples (20 µL) with 280 µL of methanol in the same plate. The blank absorbance was subtracted from the sample reading to obtain DPPH radical scavenging activity. Ascorbic acid was used as the positive control to prepare the calibration curve. The results were expressed as ascorbic acid equivalents.

4.3.9 Statistical Analysis

For beetroot and arugula samples, 3 replications were used then all analysis was conducted in triplicate. Data were analyzed by one-way analysis of variance (ANOVA) using JMP Pro12 statistical software (SAS, NC, USA). Significance differences between means were observed by Tukey HSD test at 95% confidence interval ($P < 0.05$).

4.4 Results and Discussion

4.4.1 Stability of nitrate and nitrite during storage

Analysis of nitrate and its reduction to nitrite at different storage temperatures was performed using reversed phase HPLC. **Figure 3** shows the formation of nitrite during the storage period, and HPLC chromatograms of beetroot and arugula. Nitrate is converted to nitrite at 25 and 4°C during storage. **Figure 4** displays the levels of nitrate and nitrite during storage in beetroot and arugula juice samples. The samples stored at room temperature showed a decrease in the mean nitrate content of beetroot juice after eight hours and a substantial loss in both juices after twenty-four hours. For beetroot juice, the mean initial level of nitrate was 4965.34±72.69 µg/mL and after storage at 25˚C for one day, it decreased to 2167.29±71.07 µg/mL (**Figure 4A**). These results are in accordance with the published literature, which found that after 1 day of storage at ambient temperature carrot, beetroot, and cabbage juices had a significant decrease in nitrate levels.[74] In the arugula juice, sample the nitrate content after one day at room temperature decreased from 6310.20±24.79 µg/mL to 3085.89±41.87 µg/mL. Under refrigerated storage at 4°C, nitrate levels were relatively stable for a one-week period in beetroot juice (**Figure 4B**). In arugula juice after four days of storage at 4°C a

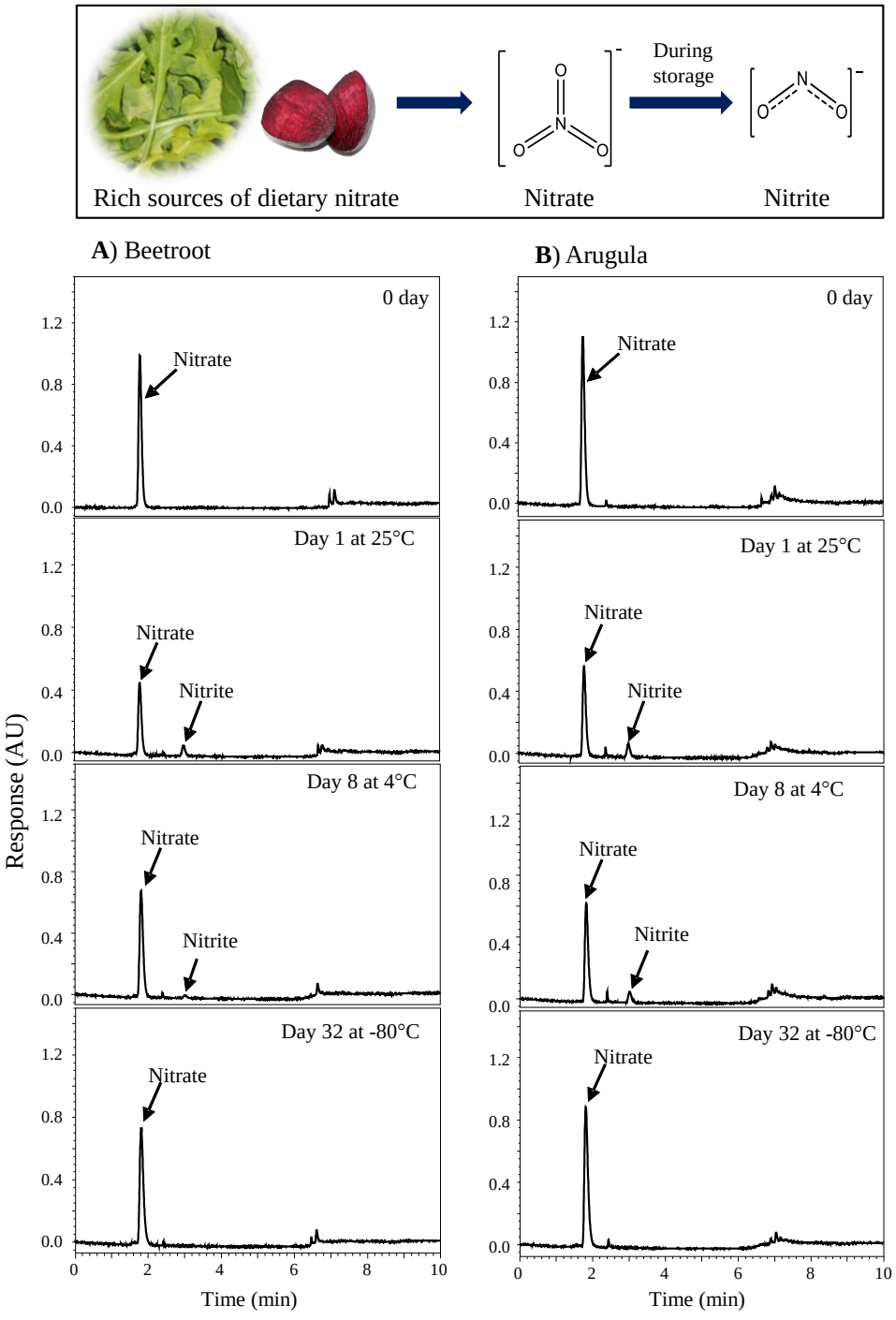

Figure 3. HPLC-UV overlay chromatograms of (A) beetroot and (B) arugula representing the reduction of nitrate to nitrite at different storage conditions.

significant difference in the nitrate content was observed. The effects of refrigeration on different crops have been shown to vary in the published literature. [37, 74, 133] Reports for arugula, different types of spinach, crown daisy, and Chinese cabbage show almost unaffected nitrate levels after one week of refrigeration.[37, 133] However, the literature shows that for juice samples of carrot, beetroot, and cabbage, 11–30% decreases in nitrate content have been observed after two days of refrigeration.[74] These results indicate that the juice matrix and the individual plant matrices may influence nitrate dynamics at refrigerated temperatures. The juice samples that were stored at -20°C or -80°C showed no degradation in nitrate levels. Conversion of nitrate to nitrite due to microbial reduction was not expected under freezing conditions and our results match the published literature.[75, 134] Previous studies showed that after a one-year storage period the levels of nitrate remained relatively stable at -20 and -30˚C.[134]

Beetroot and arugula juice did not show any detectable nitrite levels in the initial samples (**Figure 4C and 4D**). After storage for 1 day at room temperature, the reduction of nitrate to nitrite occurred and increased nitrite levels in beetroot (904.12±14.83 µg/mL) and in arugula juices (1900.31±49.17 µg/mL) were observed. These results support a previous study that showed an increase in nitrite for nitrate-rich vegetables after 24-48hrs of ambient temperature storage.[74] For beetroot juice samples stored at 4°C, the nitrite level was 164.34±38.47 µg/mL after 8 days and 1531±44.06 µg/mL after 16 days. In arugula juice, nitrite levels 48.54±21.99 µg/mL at four days. In leafy greens, an increase in nitrite after 3–4 days of storage at ambient temperature has been observed.[133] After 8 and 16 days, the levels were found to be 1361.64±192.03 µg/mL

and 1754.19±67.07 µg/mL, respectively. These results demonstrated that the storage conditions have a major effect on the stability of nitrate.

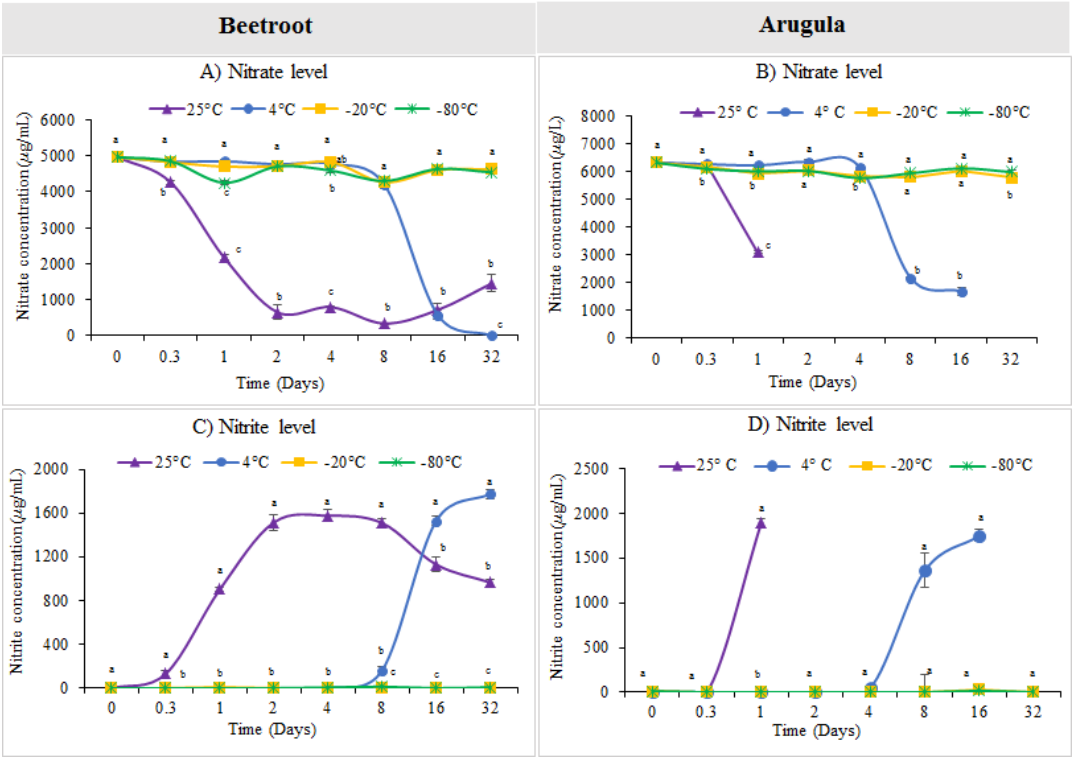

Figure 4. Levels of nitrate in (A) beetroot and (B) arugula and the reduction of nitrate to nitrite during storage in (C) beetroot and (D) arugula juice samples during storage at four different temperatures for 32 days measured by high pressure liquid chromatography. Data are expressed as mean of three replicates (± standard error). All value are given in µg/mL and significant differences were analyzed for different temperatures at each time point. Means with different letters within columns indicate significantly different value (P<0.05).

4.4.2 Identification of phenolic compounds by UHPLC-HR-ESI-QTOF-MS

The stored beetroot samples were analyzed by UPLC-HR-QTOF-MS in positive ionization mode. **Figure 5 A, B and C** represents the total ion chromatogram (TIC) and

extracted ion chromatogram (EICs) of betacyanins from beetroot juice samples at 0 day, after 32 days at 25°C, and after 32 days at -80°C, respectively.

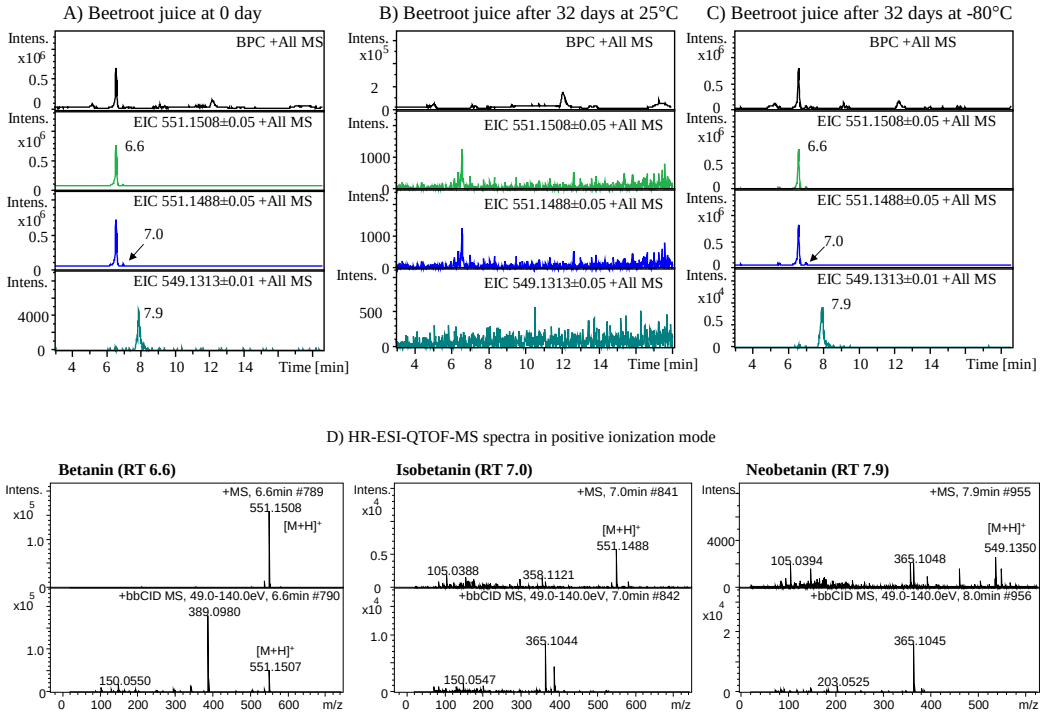

Figure 5. Total ion chromatogram and extracted ion chromatograms (EIC) of betacyanins from beetroot juice sample at (A) 0 day and (B) degradation of betacyanins' after 32 days at 25°C and (C) representing the betacyanins stability after 32 days at -80˚C. D) Tandem mass spectra of three betacyanins found in beet juice at 0 day sample analyzed by HR-ESI-QTOFMS in positive ionization mode.

Results demonstrated that the beetroot juice contained betacyanin pigment at 0 day and after 32 days at -80°C (**Figure 5 A and C)** while samples stored at room temperature showed degradation of betacyanins (**Figure 5 B**). **Figure 5 D** represents the tandem mass spectra of identified pigment in beetroot juice in the 0 day sample. The peaks eluted at RT 6.6, 7.0, and 7.9 min were identified as betanin (*m/z* 551.1508),

isobetanin (*m/z* 551.1488), and neobetanin (*m/z* 549.1351), respectively having accurate mass errors of 0.18, -3.4, and -0.2 ppm, respectively (**Table 6**).

Table 6. Identified betacyanins from beetroot juice and phenolics from arugula juice using LC-HR-ESI-QTOF-MS in positive ionization mode.

	RT[a] (min)	Compound	Molecular formula	Experimental MS fragment *m/z*[b]	Theoretical MS fragment *m/z*	Mass error (ppm)[c]
Beet root juice	6.6	Betanin	$C_{24}H_{27}N_2O_{13}$	551.1508	551.1507	0.18
	7.0	Isobetanin	$C_{24}H_{27}N_2O_{13}$	551.1488	551.1507	-3.4
	7.9	Neobetanin	$C_{24}H_{25}N_2O_{13}$	549.1350	549.1351	-0.2
Glucosides from arugula juice	9.2	Quercetin-3,3′,4′-triglucoside	$C_{33}H_{40}O_{22}$	798.2138	789.2083	6.9
	10.1	Kaempferol-3,4′-diglucoside	$C_{27}H_{30}O_{16}$	611.1648	611.1606	6.7
	10.5	Isorhamnetin-3,4′-diglucoside	$C_{28}H_{32}O_{17}$	641.1762	641.1712	7.7
	11.3	Quercetin-3,4′-diglucoside-3′-(6-sinapoyl-glucoside)	$C_{44}H_{50}O_{26}$	995.2730	995.2663	6.7
	12.4	Isorhamnetin-3-glucoside	$C_{22}H_{22}O_{12}$	479.1176	479.1184	-1.6
	13.1	Quercetin-3-(2-sinapoyl-glucoside)-3′-(6-sinapoyl-glucoside)-4′-glucoside	$C_{55}H_{60}O_{30}$	1201.3261	1201.3242	1.5
Aglycone from arugula juice	14.1	Quercetin	$C_{15}H_{10}O_7$	303.0505	303.0499	1.8
	15.9	Kaempferol	$C_{15}H_{10}O_6$	287.0551	287.0550	0.2
	16.5	Isorhamnetin	$C_{16}H_{12}O_7$	317.0664	317.0655	2.5

The LC elution orders are in accordance with published literature.[25] This suggested that during storage, betacyanin degradation occurs, which is responsible for the lower antioxidant activity of beetroot juice. Our results are consistent with the results

of previous research, where degradation of betacyanins the main phenolic compounds in beetroot juice cause the reduction in antioxidant activity.[135]

The analysis of phenolic compounds from arugula juices was also performed by UHPLC-HR-ESI-QTOF-MS in positive ionization mode. The presence of major flavonoid glucosides and their aglycone moieties in arugula juice is presented in **Table 6**. Arugula juice was rich in flavonoids, especially quercetin glucoside derivatives. **Figure 6 A, B and C** represents the TIC and EICs of flavonoid glucosides from arugula juice at 0 day, after 32 days at 25°C, and after 32 days at -80°C, respectively. Arugula juice contained flavonoid glucosides at day 0 and after 32 days at -80°C. In samples stored at room temperature, there no flavonoid glucoside peaks were observed after 32 days (**Figure 6 B**). This showed that the breakdown of flavonoid glucosides occurred during storage. **Figure 7 A** represents the tandem mass spectra of identified flavonoid glucosides from arugula juice at 0 h and -80°C. The first peak, which eluted at RT 9.2 min, showed an accurate mass of precursor ion at *m/z* 789.2138 $[M+H]^+$ (mass error 6.9 ppm). The precursor ion loss glucose moiety (-162 Da) gives product ion peak at *m/z* 627.1595 $[M+H-162]^+$ which further undergoes loss of one glycose (-162 Da) mass unit to give product ion at *m/z* 465.1058 $[M+H-324]^+$.

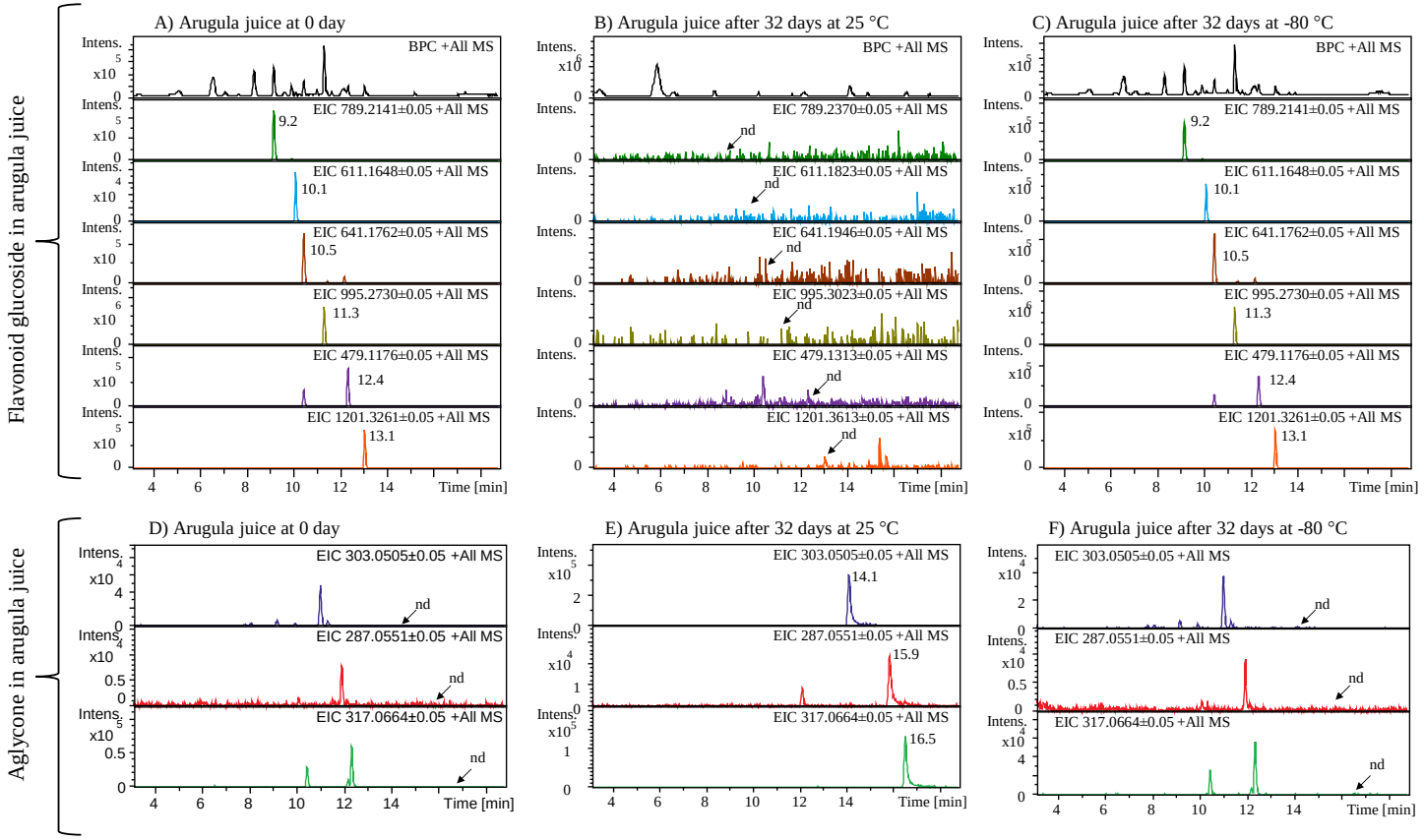

Figure 6. Total ion chromatogram (TIC) and extracted ion chromatograms (EIC) of flavonoid glucosides from arugula juice samples at (A) 0 day, (B) breakdown of flavonoid glucosides after 32 days at 25˚C and (C) representing the flavonoid glucosides' stability after 32 days at -80˚C. The TIC and EICs of aglycone (D) represents the absence of aglycone at 0 day (E) shows the prominent peaks of aglycone due to breakdown of flavonoid glucosides after at 32 days and (F) display the no aglycone peak after 32 days at -80˚C analyzed by HR-ESI-QTOFMS analysis in positive ionization mode.

The product ion (*m/z* 465.1058) underwent further loss of -162 Da mass unit to give an aglycone base peak *m/z* 303.0525 $(Y_0)^+$, which corresponds to the quercetin aglycone moiety. The fragmentation pattern obtained from HR-ESI-QTOF-MS corresponds to the quercetin-3,3,4′ -triglucoside. Our results are consistent with the results of published literature.[77, 79] Similarly, the peak that eluted at RT 10.1 and RT 10.5 min were identified as kaempferol-3,4′-diglucoside (*m/z* 611.1648 $[M+H]^+$, mass error 6.7 ppm) and isorhamnetin-3,4′-diglucoside (641.1762 $[M+H]^+$, mass error 7.7 ppm).

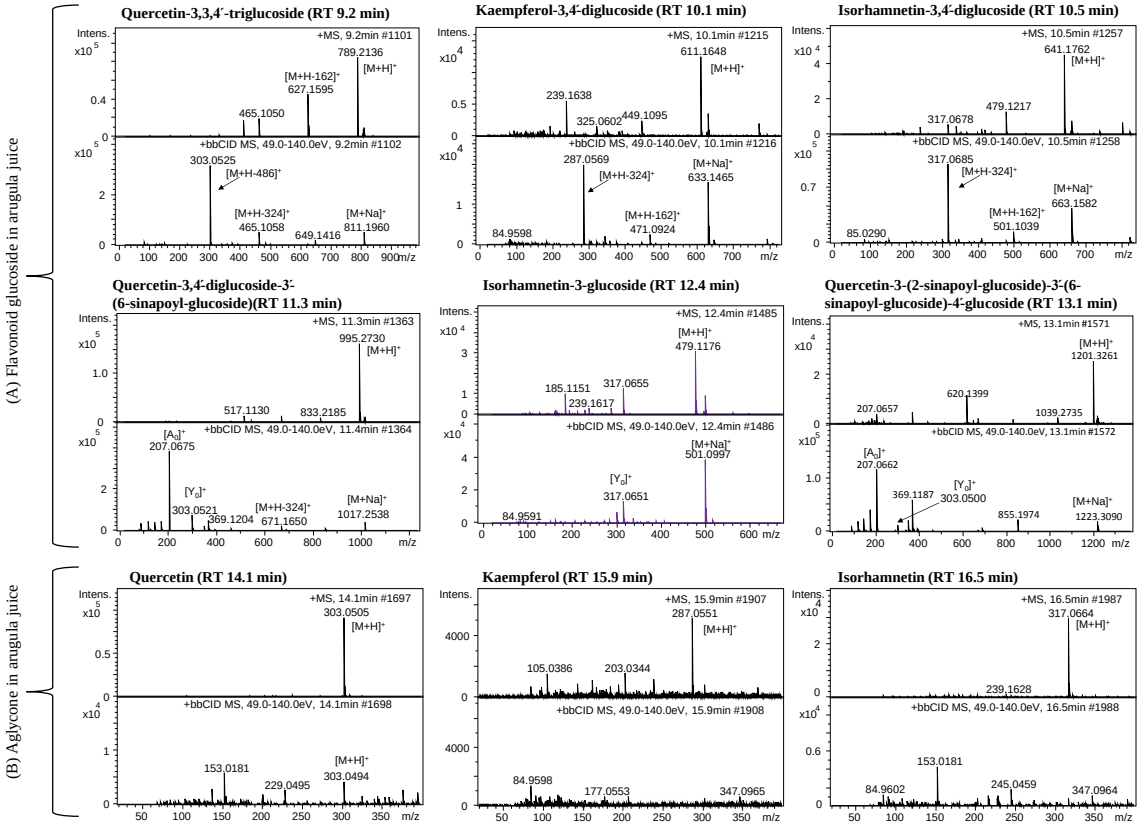

Figure 7. Tandem mass spectra of (A) flavonoid glucosides and (B) aglycone identified in arugula juice at 0 day and 32 days, respectively, by HR-ESI-QTOFMS analysis in positive ionization mode.

The precursor ions of both eluted peaks lost two glucose moieties to give prominent aglycone base peaks at *m/z* 287.1058 $(Y_0)^+$ and *m/z* 317.1221 $(Y_0)^+$, which correspond to the kaempferol and isorhamnetin aglycone moiety, respectively.[79, 80]

The peak eluted at RT 11.3 min shows an accurate mass value at *m/z* 995.2730 $[M+H]^+$ having a mass error 6.7 ppm. The precursor ion undergoes loss of neutral mass unit (-162 Da) to give a product ion at *m/z* 833.2185 $[M+H-162]^+$, which further lost -162 Da mass unit to give a product ion at *m/z* 671.1650 $[M+H-324]^+$ and a base product ion peak at *m/z* 303.0521 $(Y_0)^+$. The product ion peak at *m/z* 369.1204 corresponds to a sinapoyl glucoside moiety which lost glucose (-162 Da) to give a prominent ion peak at *m/z* 207.0675 $[Y_0]^+$, which represents sinapic acid. Thus, on the basis of tandem mass spectrum analysis and the available literature, the present peak was identified as quercetin-3,4′-diglucoside-3′-(6-sinapoyl-glucoside).[77, 79]

The peak that eluted at RT 12.4 min displays a precursor ion at *m/z* 479.1176 $[M+H]^+$ (mass error -1.6 ppm) and its sodium adduct at *m/z* 501.0997 $[M+Na]^+$. It lost one hexosyl group (-162 Da) to give a predominant peak at *m/z* 317.0651 $[M+H-162]^+$ which resembled the presence of isorhamnetin aglycone moiety $(Y_0)^+$. Thus the present peak was identified as isorhamnetin-3-glucoside.

Similarly, the peak that eluted at RT 13.1 min shows accurate mass at *m/z* 1201.3261 $[M+H]^+$ (mass error 1.5 ppm) and its sodium adduct at *m/z* 1223.3090 $[M+Na]^+$. The precursor ion give a product ion peak at *m/z* 1039.4531 $[M+H-162]^+$. +bbCID spectrum shows a prominent product ion peak at *m/z* 369.1189 and at *m/z* 207.0662 which corresponds to sinapoyl glucoside and sinapic acid respectively. The

peak that eluted at *m/z* 303.0500 $(Y_0)^+$ represents the quercetin aglycone moiety. The fragmentation pattern of the peak eluted at RT 13.1 min was similar to that in the previous report, and thus the present peak was identified quercetin-3-(2-sinapoyl-glucoside)-3′-(6-sinapoyl-glucoside)-4′-glucoside.[79, 80]

Figure 6 D, E and F displays the TIC and EICs of aglycone from arugula juice at day 0, after 32 days at 25°C, and after 32 days at -80°C obtained from HR-ESI-QTOFMS analysis in positive ionization mode. The TIC's were screened for phenolic compounds, flavonoid glucosides and aglycones. In 0-day samples, only flavonoid glucosides were observed whereas at 32 days, degradation products of flavonoids aglycones were found. For instance, peaks eluted at RT 14.1, 15.9 and 16.5 min matched to aglycone precursor ion of quercetin (*m/z* 303.0505, mass error 1.8 ppm), kaempferol (*m/z* 287.0551, mass error 0.2 ppm) and isorhamnetin (*m/z* 317.0664, mass error 2.5 ppm) respectively (**Figure 7 B).** The presence of aglycone peaks at 32 days provides evidence that the flavonoid glucosides breakdown to their respective aglycone moieties during storage. Our results are in agreement with previous finding in orange juice which showed that during storage, an increase in the concentration of free acids such as ferulic acid and *p*-coumaric acid occurred. This was due to the release from their bound form, which led to the increase in the antioxidant activity. [136] Similarly, an increase in antioxidant activity after a 2 month storage was also reported in tomato juice. [137]. **Figure 8** represents the structures of the identified compounds from beetroot and arugula by UPLC-HR-QTOF-MS.

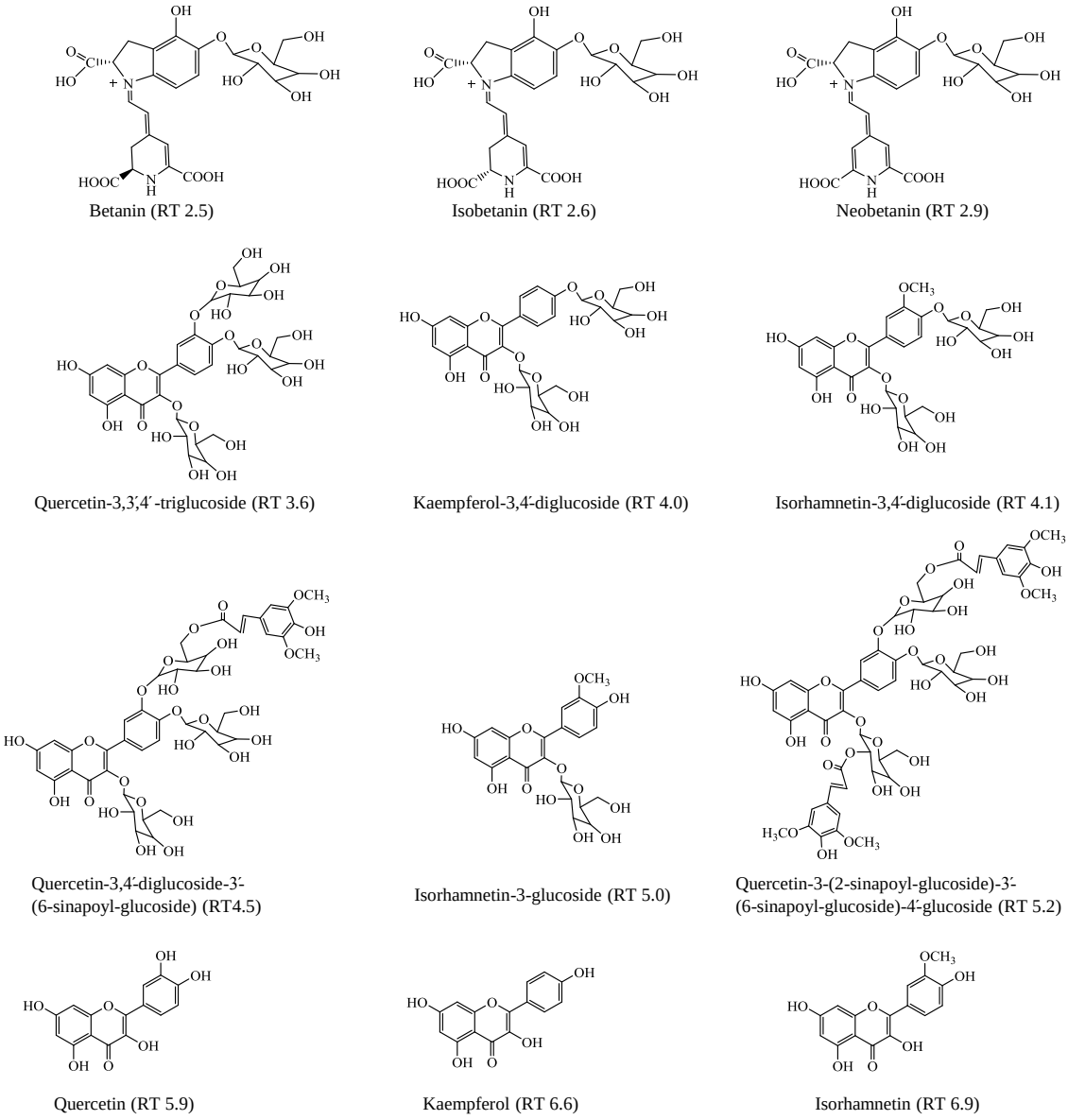

Figure 8. Structure of the identified compounds from beetroot and arugula juices.

4.4.3 Total phenolics and DPPH radical scavenging activity

The Folin-Ciocalteu (FC) method was used to evaluate the total phenolic content in juices; phenolics play important roles in counteracting reactive oxygen species and functional properties of juices. The beetroot and arugula juices were rich in phenolic compounds (**Figure 9**).

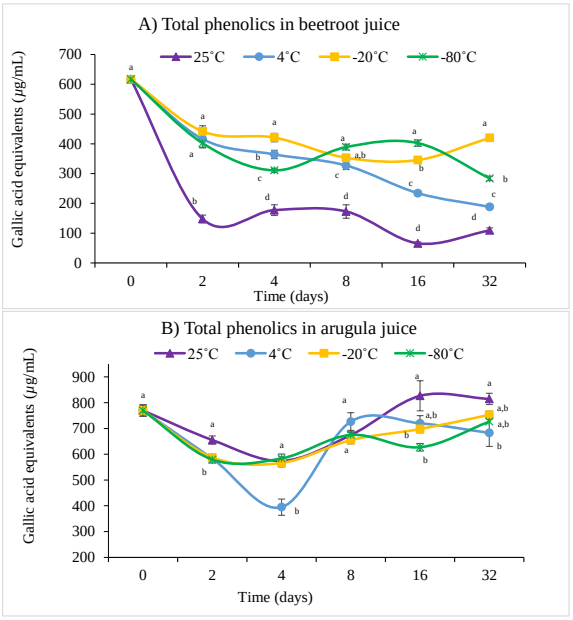

Figure 9. Stability of total phenolics compounds in (A) beetroot and (B) arugula juice stored at different temperatures over a 32-day period. Data are expressed as mean of three replicates (± standard error). All values are given in µg/mL and significant differences were analyzed for different temperature at each time point. Means with different letters within columns indicate significantly different value (P<0.05).

Beetroot juice, the phenolic contents decreased with storage time but in arugula juice the phenolic contents increased at 16 days (826.58± 58.36 µg/mL) and 32 days (814.57± 22.10 µg/mL) compared with at 0 day (769.54±21.63 µg/mL). The samples

stored in -20 and -80 °C showed similar activity at 0 and 32 days. To understand the health-promoting properties of vegetable juices, evaluation of functional properties is more critical. In general, antioxidants protect the quality of foods by retarding oxidative breakdown of lipid components. Natural antioxidants such as nonenzymatic dietary components are not specific but can scavenge organic and inorganic radicals. These antioxidants are found in numerous plant materials and commonly include phenolics, flavonoids, ascorbic acid and vitamin E.

In the present study, DPPH free radical assay was used to analyze the juice's quality; this assay is based on the reactivity of the free radical and the hydrogen donors including phenolics compounds. Antioxidants react with DPPH, which is a nitrogen-centered radical with a characteristic absorption at 517 nm, and convert it to 1,1,-diphenyl-2-picryl hydrazine, due to its hydrogen donating ability at a very rapid rate.[138] The degree of discoloration indicates the scavenging potentials of the antioxidants present in the vegetable juices. **Figure 10** represents the free radical scavenging activity of beetroot juice and arugula juice stored at different temperatures for 32 days. Free radical scavenging activity decreased with the storage time in beetroot juice. Juice stored at 4°C showed a decrease in activity after 48 h while minimum activity was observed at 32 days in all stored samples. This may be due to the degradation of betacyanin pigment in beetroot juice (**Figure 5B**), which was responsible for higher activity of beet juice. Interestingly, arugula juice samples stored at 4 °C showed higher activity at 16 days (571.12± 33.75 µg/mL) and 32 days (510.65± 10.76 µg/mL) than samples analyzed at 0 day (380.84± 5.85 µg/mL) while samples stored in -20 and -80°C showed similar

activity at the 0 and 32 days. The higher activity may be due to breakdown of higher phenolic or flavonoid glucosides to their lower phenolic compounds or in their aglycone moieties respectively (**Figure 6D**).

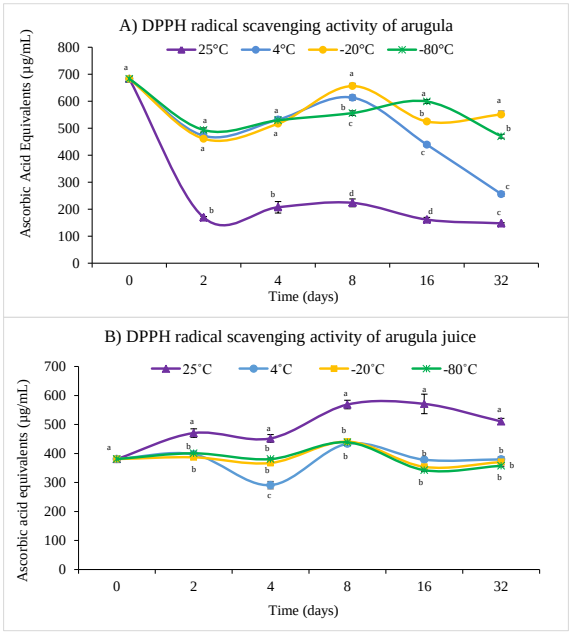

Figure 10. Stability of DPPH radical scavenging activity in (A) beetroot and (B) arugula juice stored at different temperatures over a 32-day period. Data are expressed as mean of three replicates (± standard error). All value are given in µg/mL and significance differences were analyzed for different temperatures at each time point. Means with different letters within columns indicate significantly different values ($P<0.05$).

The presence of a sugar moiety significantly decreases the antioxidant activity of the flavonoids. For instance, quercetin oxidizes fastest as compare to quercetin and rutin, the oxidation rate of quercetin decreased as the substituent at C_3 became a poorer leaving group.[139] Moreover, disaccharide containing flavonoids exhibit lower activity compared

to monosaccharides.[140] Thus, the arugula juice at 32 days exhibited same or higher radical scavenging activity.

4.5 Conclusion

In conclusion, we observed that storage temperature and period had a significant effect on the levels of nitrate and nitrite. At 25˚C, the reduction of nitrate started after one day of storage, which caused an increase in nitrite content. At 4°C, nitrate was stable for a longer period without any noticeable nitrite levels. For beetroot juice, the degradation of nitrate was not observed at 8 days while arugula degradation was observed after 4 days. Storing samples at frozen conditions of either -20 °C or -80 °C prevented the conversion of nitrate to nitrite in the juices of both vegetables even after a one-month storage period. Refrigeration may help prevent a decrease in nitrate levels in raw vegetable juices over a short time frame. For long term storage, however, freezing is required to prevent a decrease in nitrate content and an increase in nitrite. The stability of betacyanins was also influence by the storage conditions. The increase in the antioxidant activity in arugula juice was observed due to the breakdown of flavonoids glucosides to their aglycone moiety or lower phenolic compounds, which was confirmed by UPLC-HR-QTOF-MS analysis. These findings may open new paths to investigate the development of functional juices that can retain nitrate and stimulate the breakdown of flavonoids glucosides to their alcyone moieties in a safe manner for cardiovascular health.

CHAPTER V

EVALUATION OF HEALTH-PROMOTING COMPOUNDS OF BEETROOT, WATERMELON, KALE, ARUGULA, AND THEIR JUICE BLENDS

5.1 Synopsis

The consumption of vegetables has been associated with a reduced risk of developing chronic diseases. These benefits have been attributed to the various bioactive compounds present in vegetables. Certain vegetables contain dietary nitrate and L-citrulline, which are precursors to nitric oxide (NO), a potent vasodilator that has benefits in cardiovascular health and sports performance. Combining these vegetables into juice blends may lead to a natural functional beverage that has beneficial levels of precursors of NO. In the present study, juices of watermelon, beetroot, kale, arugula, and six different juice blends were evaluated for their bioactive composition, amino acid profile, and antioxidant activity. All vegetables were purchased from three different stores, juiced using an Omega HD juicer, and mixed into six different juice blends (B-1 to 6). Beetroot juice had the highest level of nitrate (4885.97±345.40 µg/mL), followed by kale (4833.08±407.46µg/mL), and arugula (3346.02±317.08 µg/mL). For the blends, B-1 and B-2 had significantly higher levels of nitrate 2826.14±224.77 µg/mL and 2612.61±203.49 µg/mL, respectively, than the other juice blends. Watermelon juice had the highest levels of L-citrulline (1279.97±24.52 µg/ mL) followed by B-6 (1001.16±35.70 µg/mL). The juices were also freeze-dried and then evaluated for their nitrate and L-citrulline content. Overall, the percentage of beetroot juice in blends affected the nitrate content while citrulline content was determined by watermelon juice.

In conclusion, the blending of vegetable juices can increase the levels of bioactive compounds and enhance the nitrate and L-citrulline contents, which may lead to improved functional properties.

5.2 Introduction

Lower overall mortality has been associated with the consumption of fruits and vegetables. A reduced risk of developing cardiovascular disease, cancer, diabetes and other age-related diseases has also been observed. [1, 2, 141] Fruit and vegetable consumption in the United States is considered low, with adults consuming fruits 1.1 times per day and vegetables 1.6 times per day. [5] The 2015-2020 Dietary Guidelines for Americans encourages the consumption of a wide variety of fruits and vegetables. [142] These dietary guidelines state that vegetable and 100% fruit juices without added sugars can contribute to consumption of the daily recommended servings of fruits and vegetables. [142]

Juices are a simple means of incorporating more vegetables into the diet. Moreover, they could also act as functional foods. These are foods that provide benefits beyond basic nutrition. [16, 143] The bioactive compounds present in commercially available and underutilized crops may help reduce the risk of chronic diseases [11, 144] The blending of different vegetable and fruit juices can improve the nutritional quality of a juice by combining various classes of bioactives. Mixing juices can also improve the acceptability of a functional beverage and help consume fruits or vegetables that are high in bioactives but have bitter or off-flavors [91, 92].

Watermelon, beetroot, and leafy greens such as kale and arugula contain various bioactive compounds. Watermelon is a cucurbit that is classified as a vegetable, but is mainly consumed as a dessert due to its sweetness.[41] Watermelon has mineral salts (K, Mg, Ca, and Fe), vitamins (A, B, C, and E), amino acids including L-citrulline and L-arginine, carotenoids and phenolics[41-43]. Beetroot, an edible taproot has received attention as a functional food. It contains dietary nitrate, betalains, hydroxycinniamic acids, and flavonoids.[28, 29] Ingestion of beetroot juice raises nitrite plasma levels due to its nitrate content.[30] Recent evidence demonstrated that dietary nitrate has cardioprotective properties and may aid in blood pressure regulation.[8, 30] Kale is a leafy green that contains a broad range of bioactive compounds including glucosinolates, vitamins, polyphenols, phenolic acids, chlorophylls, carotenoids, and amino acids.[49, 50, 54] Arugula, has a very distinct peppery taste and pungent aromas.[31, 32] It contains high nitrate, vitamin C, and fiber and possesses a variety of phytochemicals such as carotenoids, glucosinolates, and flavonoids.[32, 36]

A functional beverage from these crops could potentially aid in nitric oxide production. Nitric oxide, a free radical produced *in vivo* has various effects on the body.[18] Importantly, it signals for vasodilation that allows for improved blood flow. Adequate function of nitric oxide is vital for cardiovascular health, especially in pre-hypertensive and hypertensive individuals.[40] Vasodilation may also have benefits for sports performance due to improved blood flow. Two pathways that lead to the production of nitric oxide are the nitric oxide synthase (NOS) dependent and NOS independent pathways, which utilize L-arginine/L-citrulline and dietary nitrate,

respectively. Beetroot juice containing dietary nitrate improves blood flow by causing vasodilation via supplementation to the NOS-independent pathway.[19-21] This pathway produces nitric oxide by the reduction of dietary nitrate to nitrite by anaerobic bacteria in the oral cavity; this nitrite is further reduced to nitric oxide. Studies using L-citrulline and L-arginine from watermelon and other sources suggest that there are also benefits in improving vasodilation through the NOS-dependent pathway.[12, 22] Nitric oxide is produced endogenously by interconversion of L-arginine to L-citrulline via the enzyme NOS in this pathway. A natural vegetable juice containing these compounds could potentially help enhance nitric oxide production, which could positively impact cardiovascular function and sports performance.

The level of dietary nitrate, L-citrulline, and other bioactives from vegetable juices should be optimized to find a blend that could have function properties for nitric oxide production. However, levels of bioactives in fresh juices that do not contain additives are not standardized [3, 10]. This is due to the fact that bioactive compounds vary based on variety, growing location, season, fertilization and other environmental factors. [145] The aim of this study was to determine levels of health promoting compounds from juices of the selected vegetables watermelon, beetroot, kale, arugula and to evaluate their optimal juice blends to maximize the levels of NO precursors such as nitrate and L-citrulline.

5.3 Materials and Methods

5.3.1 Chemicals

Sodium nitrate, L-ascorbic acid, gallic acid, analytical standard grade amino acids, sucrose, glucose, fructose, 2,2-Diphenyl-1-picrylhydrazyl, Folin-Ciocalteu (FC) reagent, metaphosphoric acid (MPA), sodium acetate trihydrate, HPLC grade acetonitrile and methanol, phosphoric acid, and drum grade methanol were purchased from Millipore Sigma (St. Louis, MO, USA). Tris (2-carboxy ethyl) phosphine hydrochloride (TCEP), L-ornithine, hydrochloric acid, sodium borate, and acetic acid glacial HPLC grade were obtained from Fisher Scientific (Fair Lawn, NJ, USA). Sodium carbonate, *O*-phthalaldehyde (OPA) and beta-alanine were from VWR (Radnor, PA, USA). Nano-pure HPLC grade water (resistivity 18.2 mΩcm) was obtained from (NANO pure, Barnstead/ Thermolyne, Dubuque, IA, USA).

5.3.2 Plant materials

Fresh beetroot (*Beta vulgaris*), watermelon (*Citrullus lanatus*), kale (*Brassica oleracea*), and arugula (*Eruca sativa*) were purchased at local grocery stores HEB, Kroger and Farm Patch (Bryan/College Station, TX, USA).

5.3.3 Processing of vegetables

Vegetables were rinsed thoroughly with tap water followed by nano-pure water. After rinsing the vegetables, inedible parts were removed. For watermelon, the rind was separated and only the flesh was used and for the leafy greens any stems or leaves that showed signs of wilting were discarded. The vegetables were then cut into small pieces and juiced individually with 3 independent replications per store for a total of nine

replicates of each juice. The juice was extracted with an 8006 Nutrition System HD Juicer (Omega, Harrisburg, PA, USA).

5.3.4 Formulation of juice blends

The vegetable juices were mixed at predetermined ratios (**Table 7**) into six juice blends per store with 3 replicates each and immediately stored at -20°C until further analysis.

Table 7 Percentage (%) of watermelon, beetroot, kale and arugula in juice blends.

Juice Blend	Watermelon	Beetroot	Kale	Arugula
Blend 1	50	50	-	-
Blend 2	50	30	15	5
Blend 3	60	30	5	5
Blend 4	70	20	5	5
Blend 5	75	15	5	5
Blend 6	80	10	5	5

5.3.5 Preparation of methanolic extracts

Methanolic extracts were prepared for DPPH assay and total phenolics assay. For each sample 2 mL of juice was diluted to 10 mL with methanol and vortexed for 2 min. to a homogenized mixture. The samples where then passed through Whatman No. 1 filter paper, 1.5 mL was placed in a microcentrifuge tube and centrifuged for 6 min. at 7826 x g. The clear supernatant was stored at -20ºC until further analysis.

5.3.6 Color measurement

Color analysis of juice blends and individual vegetable juices was measured with a Minolta CR-400 Chroma Meter (Konica Minolta Sensing, Inc., Osaka, Japan). Calibration of the colorimeter was performed using a white calibration plate before measurement. Color was expressed in Commission Internationale de l'Eclairage (CIE), L*a*b* coordinates. In which L* measures lightness (+ = lighter, - = darker), a*measures red/green (+ = redder, - = greener), b* measures yellow and blue (+ = yellower, - = bluer). Chroma (C*), hue angle (h) and total color difference (ΔE) were also evaluated. For the evaluation of total color different pure vegetable juices were used as standards and blends as samples to determine color difference between and vegetable and blended juices. All color measurements were recorded in triplicate for all samples.

5.3.7 Total soluble solids, pH and titratable acidity

A hand refractometer (American Optical Corp., South Bridge, MA, USA) was used to measure total soluble solids (TSS). Titratable acidity of the juices was measured using a DL 22 Food and Beverage analyzer (Mettler Toledo, Columbus, OH, USA). pH measurements were analyzed using a Mettler Toledo EL Education Line pH Meter. All measurements were evaluated in triplicate for all samples.

5.3.8 Quantification of nitrate

Nitrate content in the fresh vegetable juices was analyzed by the HPLC method according to Chou et al. 2003 [146] with slight modification. Juice samples were diluted with nano-pure water centrifuged and passed through 0.45 cellulose filter before analysis. Chromatographic separations of nitrate were performed by Waters 1525 HPLC

series (Milford, MA, USA) equipped with a 2996 photodiode array detector and Waters 717 plus autosampler using a Zorbax eclipse plus C_{18} ODS column (5 micron, 250 mm × 4.6 mm) (Agilent, Santa Clara, USA). Isocratic mobile phase 0.03M phosphoric acid: acetonitrile (90:10) with a flow rate of 0.6 mL/min was used. Run time consisted of 15 min and detection was carried out with a photo diode array (PDA) detector at 210 nm. A sodium nitrate standard was prepared in nano pure water at various concentrations to obtain a calibration curve for quantification of nitrate.

5.3.9 Analysis of Ascorbic Acid

Juice samples, were extracted with 3% MPA according to Chebrolu et al. 2012 for the analysis of ascorbic acid [147]. For analysis a Thermo Finnigan, Spectra system (Waltham, MA, USA), with a PDA detector (spectra system UV6000 LP) coupled with a quaternary pump system P4000 and an AS3000 auto sampler was used. A C_{18} Gemini column (250mm × 4.6 mm), an isocratic mobile phase of 0.03M phosphoric acid with a flow rate 0.7 mL/ min and a run time of 15 minutes were used. Detection was carried out at 243 nm. For determination of total ascorbic acid TCEP was added to samples with a 1:1 ratio and incubated at room temperature for 1 hour. To calculate dehydroascorbic acid, the ascorbic acid content was subtracted from the total ascorbic acid content. Quantification was determined with a calibration curve of ascorbic acid standard, obtained by running standard at different concentrations.

5.3.10 Total phenolics assay

Total phenolic content of samples was determined using the Folin-Ciocalteu (FC) method [131]. Juice samples were diluted with methanol and vortexed for 2 minutes, then

filtered with Whatman No. 1 filter paper. In a 96-well plate, a known amount of sample was adjusted to 180 µL with nano-pure water. Then 40 µL of 25% solution FC reagent was added and incubated at room temperature for 10 minutes. This was followed by the addition of 50 µL of sodium carbonate and incubation at room temperature for 20 minutes. Gallic acid standard was used for the calibration curve. At 760 nm the absorbance of blue color was measured using a KC-4 Microplate Reader (BioTek Instruments, Winooski, VT, USA).

5.3.11 2,2-Diphenyl-1-picrylhydrazyl (DPPH)

Methanol vegetable juice extracts were used for the DPPH radical-scavenging using the method described by Jayaprakasha et al., [132] with slight modifications. Extracts were pipetted into 96-well micro plates with the total volume of each well adjusted to 100 µL with MeOH. An aliquot of 180 µL of DPPH solution was added to the standard and sample wells, after a 20 minute incubation in the dark; the absorbance was measured at 515 nm. For pigmented samples, blanks without DPPH were used and the absorbance of the blanks subtracted from the sample reading. Ascorbic acid was used as a standard.

5.3.12 Quantification of amino acids

Determination of amino acids was carried out by precolumn derivatization with OPA according to Wu and Menninger with slight modifications, and then analyzed with a HPLC system. The system comprised of a Perkin Elmer Series 200 binary pump and autosampler (Perkin Elmer Life and Analytical Sciences, Shelton, CT, USA). A Gastorr TG-14 inline HPLC mobile phase degasser (FLOM USA, San Diego, CA, USA) and an Eppendorf TC-50 controller with a CH-30 column heater (Eppendorf, Westbury, NY,

USA). Detection was performed by an Agilent Technologies 1260 Infinity fluorescence detector controlled by an Agilent Instant Pilot model G4208A (Agilent Technologies, Santa Clara, CA, USA). The system was controlled by a PE Nelson 900 interface and a PE Nelson 600 Link box (Perkin Elmer Life and Analytical Sciences, Shelton, CT, USA). Perkin Elmer TotalChrom version 6.3.2. software was used for instrument control and data acquisition. The mobile phase consisted of a 20 mM sodium acetate buffer pH 5.4 (A) and an acetonitrile/methanol/water (B) mixture. Elution was performed in gradient. The excitation and emission of the fluorescence detector was set at 340 nm and 455 nm, respectively, which is optimal for OPA-derivatives. Serial dilutions of amino acids were run and regression equations obtained.

5.3.13 Analysis of sugars by HPLC

Quantification of glucose, sucrose, and fructose was analyzed using HPLC and RI detector using method by Yoo et al. (2012) with slight modifications.[148] Before analysis samples were diluted with nano-pure water centrifuged and filtered, and then injected into an HPLC system consisting of a series 200 autosampler and binary pump (Perkin Elmer LC-200, Norwalk, CT, USA). The HPLC system was connected to a refractive index-150 detector (System Spectra), a column heater (Jones chromatography, Lakewood, CO, USA) and a carbohydrate column equipped with guard cartridge. An isocratic mobile phase consisting of water was degassed using degasser (Gastorr TG-14). For calculations, external standard calibration curves of glucose, sucrose and fructose were used.

5.3.14 Nitrate and L-citrulline content in freeze-dried powder

A preliminary study was conducted to analyze the nitrate content in the free dryer vegetables juices and their blends. Each juices sample was initially frozen at -80 °C for overnight. The frozen juice samples were freeze-dried using a lyophilizer (Labconco FreeZone, Kansas City, MO, USA) and stored at -80°C until further analysis. Then, 20 mg of each freeze-dried powder was dissolved in the nano-pure water and vortexed for 2 min to obtain homogenized sample. HPLC analysis for nitrate was performed according to the HPLC method described in section 5.3.12. The L-citrulline content was analyzed by HPLC-FLD according to method in section 3.3.8.

5.3.15 Statistical analysis

All the results were conducted with three independent replications in triplicates, analyzed with JMP Statistical Discovery (SAS) Pro.v.12.0 software package, and processed by one-way analysis of variance (ANOVA) to evaluate significant difference ($p<0.05$) and Tukey's HSD (honest significant difference) test for comparison of sample means.

5.4 Results and Discussion

5.4.1 Juice yield

Juice yield was affected by the food matrix of the different fresh vegetables **(Table 8)**. Watermelon which contains high water content showed the highest percent yield of all the vegetables 84.10± 2.06 (Table 8). Arugula and kale followed with % yields of 67.57±3.53 and 60.90±2.66, respectively. Beetroot gave the lowest % yield of 55.36± 2.33.

Table 8 Juice Yield for beetroot, watermelon, kale, and arugula.

Juice	Vegetable							
	Beetroot		Watermelon		Kale		Arugula	
% Yield	84.10	±2.06	55.36	±2.33	60.90	±2.66	67.57	±3.53

±Standard error calculated from 9 replicates (3 replicates per store).

5.4.2 Quality parameters

Total soluble solids (TSS), pH, and % titratable acidity (%TA) are important parameters in evaluating juice quality. **Table 9** represents the quality parameters of individual vegetables juices and their blends. The TSS levels varied with the different blends, beetroot juice showed the highest TSS content ranging from 9.18% to 11.84% for store S-1 to S-3 respectively (Table A-3). Watermelon juices also have higher TSS value ranging from 8.74% to 9.23%. The arugula juice has the lowest TSS ranging from 4.54%to 6.61%. Among the blends, blend-1 (beetroot: watermelon) had the highest TSS contents (9.13% - 10.01%). No significant difference was observed in pH and titratable acidity among different blends, except the kale showed higher acidity ranging from 0.2% to 0.28 %.

5.4.3 Color

Color is a measure of juice quality and is considered a crucial parameter for consumer acceptability. [28] Table A-4 illustrates the color values for the pure and blended juices for all data combined and Table A-5 for the individual stores. The chroma (C*) and hue angle (h*) offer further description of the spatial color distribution by measuring color appearance properties.[104] Chroma (C*) displays the purity and intensity of color saturation.[149] Among the pure vegetable juices, watermelon contained the highest C* value 20.74±0.41 followed by arugula 10.99±0.46 then kale 10.64±0.26. Beetroot juice

had the lowest C* value and as the amount of beetroot juice decreased in the blends the C* values increased signifying an increase in color purity.

Table 9 Quality parameters for beetroot, watermelon, kale arugula, and blended juices of pH, total soluble solids, and % titratable acidity.

Juice	TSS	pH	% TA
Beet	8.83 ± 0.08^{a}	5.97 ± 0.03^{ab}	0.12 ± 0.00^{cd}
Watermelon	10.48 ± 0.23^{c}	6.26 ± 0.01^{e}	0.13 ± 0.00^{e}
Kale	7.10 ± 0.09^{d}	5.97 ± 0.02^{de}	0.23 ± 0.01^{a}
Arugula	5.63 ± 0.22^{e}	6.18 ± 0.03^{abc}	0.17 ± 0.00^{b}
Blend 1	9.67 ± 0.09^{b}	6.29 ± 0.07^{a}	0.13 ± 0.00^{cd}
Blend 2	8.85 ± 0.05^{c}	6.32 ± 0.06^{a}	0.14 ± 0.00^{c}
Blend 3	9.01 ± 0.06^{c}	6.09 ± 0.02^{bcd}	0.13 ± 0.00^{cd}
Blend 4	8.93 ± 0.07^{c}	6.06 ± 0.03^{cd}	0.13 ± 0.00^{cde}
Blend 5	8.88 ± 0.08^{c}	6.01 ± 0.03^{cd}	0.13 ± 0.00^{cde}
Blend 6	8.88 ± 0.08^{c}	6.01 ± 0.03^{de}	0.13 ± 0.00^{de}

TSS= Total soluble solids, represented in (Brix^{o}). % TA= Percent titratable acidity, represented in citric acid equivalents. Different letters denote a significant difference between values at $*p \leq 0.05$. Samples expressed as mean of 9 replicates (3 replications per store) ± SE.

The hue angle for the juices increased as compared to the pure juices with B3 having the highest value 344.91±1.40 indicates a hue between red (0) and blue (240). Our results are in agreement with previously reported hue angle value of 358.9 for red beetroot juice.[124] Table A-5 represents the color variation among the vegetables juices obtained from different store. The ΔE takes into account the differences between L*, a*, and b* values for a standard and sample to yield the total difference between two colors. In this experiment, the pure vegetable juices were used as standards to calculate the differences between the blends and pure juices results are represented in Table A-6. All blends

showed the lowest color differences when compared to beetroot juice as compare to the other pure samples. This indicates that the proportion of beetroot juice played a major role in influencing the color of the blend.

5.4.4 Quantification of nitrate

Green leafy vegetable and roots are rich sources of dietary nitrate (NO_3^-), among the leafy vegetables and roots, arugula, spinach, radish, and beetroot are considered to have high nitrate concentrations.[8] The levels of nitrate in different vegetable and juice blends were measured by reverse phase HPLC-UV. Beetroot possessed the highest nitrate content (4885.9±345.4 µg/mL), followed by kale (4833.08±407.5 µg/mL), and arugula (3346.02±317.1 µg/mL). Our results for beetroot and leafy greens are in accordance with the previously reported literature.[8] Watermelon had the lowest nitrate content (110.34±25.2 µg/mL). In juice blends, B-1 had the highest nitrate content which is probably due to a higher concentration of beetroot juice as compared to the others blends. Nitrate levels decreased as the beetroot juice percentage decreased and the watermelon content increased. Therefore, B-6 had the lowest nitrate levels (954.50±65.9 µg/mL) due to a higher percentage of watermelon juice (**Figure. 11**). The nitrate levels varied among the vegetables which are influence by the environment, genetic factors and agricultural practices. Organically grown vegetables have lower nitrate contents than conventionally grown vegetables.[106, 108] Nitrate levels were also observed vary among the different stores (Figure A-1).

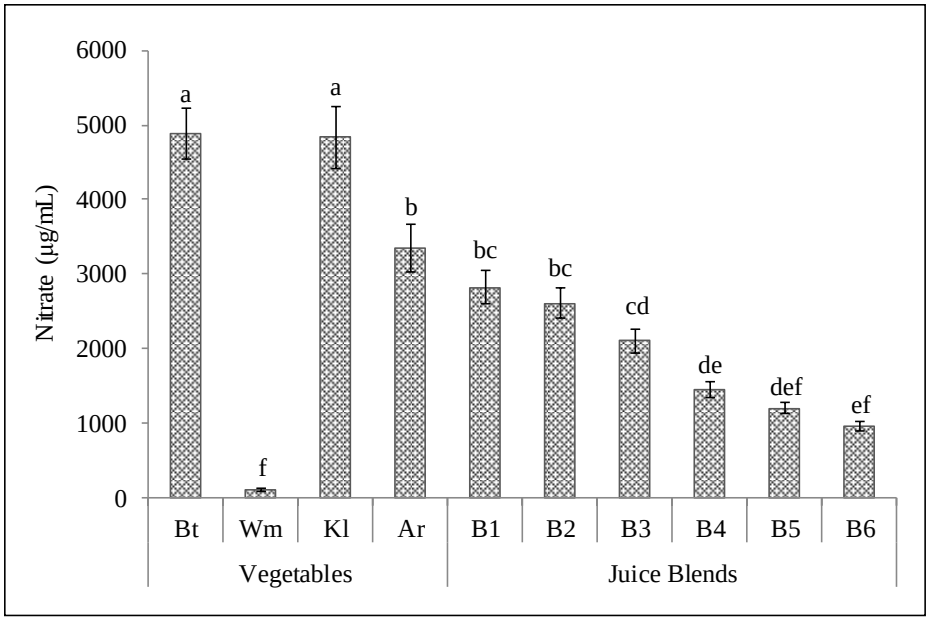

Figure 11. Nitrate content measured by high pressure liquid chromatography for beetroot, watermelon, kale, arugula and blended juices. Abbreviations represent Bt-beetroot, Wm-watermelon, Kl-kale, Ar-arugula, B1-blend 1, B2-blend 2, B3-blend 3, B4-blend 4, B5- blend 5, and B6-blend 6. Different letters denote a significant difference between values at *p≤0.05. Samples expressed as mean of 9 replicates (3 replications per store) ±SE.

5.4.5 Ascorbic acid, dehydroascorbic acid and total ascorbic acid

Ascorbic acid is a water-soluble natural antioxidant with a potent free radical scavenging activity is which rapidly oxidized in the presence of oxygen and heat. Since juice processing may affect the levels of ascorbic acid in the different blends, it was essential to determine their content in the processed juice.

Figure 12A represents the ascorbic acid content in different vegetables and juice blends. Results demonstrated that ascorbic acid content varied by vegetable and by their juice blends. Arugula juice has significantly highest ascorbic acid content, 78.84±12.6 µg/mL followed by watermelon (30.43±1.7 µg/mL), kale (14.92±2.2 µg/mL) and beetroot juice (0.86±0.2 µg/mL). All the blends (B1-6) had lower ascorbic acid. Our

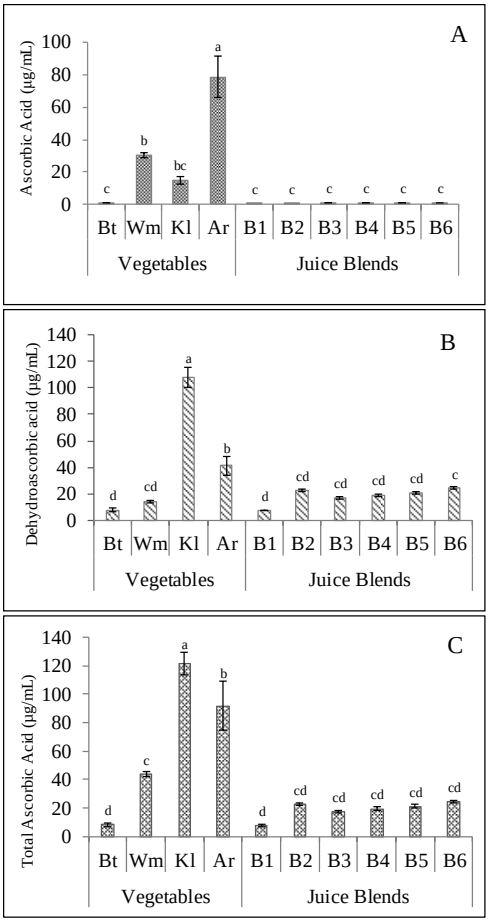

Figure 12. Ascorbic Acid, dehydroascorbic acid, and total ascorbic content measured by high pressure liquid chromatography for beetroot, watermelon, kale, arugula, and blended juices. A) Ascorbic acid, B) dehydroascorbic acid, C) total ascorbic acid. Abbreviations represent Bt-beetroot, Wm-watermelon, Kl-kale, Ar-arugula, B1-blend 1, B2-blend 2, B3-blend 3, B4-blend 4, B5- blend 5, and B6-blend 6. Different letters denote a significant difference between values at $*p \leq 0.05$ Samples expressed as mean of 9 replicates (3 replications per store) ± SE.

results are in agreement with the previously reported literature.[36, 150] **Figure 12B** displays the results of an oxygenated form of ascorbic acid, dehydrogenated ascorbic acid (DHA) of different crops and their blends. Interestingly, kale showed a significantly higher DHA level among all analyzed crops (107.58±7.6 µg/mL). Similarly, DHA content in arugula, watermelon and beetroot were found to be 41.09±7.5 µg/mL, 14.34±1.0 µg/mL and 7.76±1.0 µg/mL, respectively. Among the blends (B1–6), DHA

content varied significantly, B-6 had the highest DHA levels (24.09±0.9 µg/mL) among the blends. This may be due to a low amount of beet root juice content (5%). B1 had the lowest level of DHA (7.34±0.4 µg/mL) due to absence of kale or arugula juice content. **Figure 12C** demonstrates the TAA content (total ascorbic acid or Vitamin C). Kale had significantly higher TAA (121.40±8.2 µg/mL), followed by arugula (91.63±17.1 µg/mL), watermelon (43.42±1.6 µg/mL) and beetroot juice (8.24±1.1 µg/mL). Interestingly, the TAA content in our study was higher than the published literature.[36, 150] This may be due to effects of cultivar or growing season. Among the blends, B-6 showed the highest TAA content, 24.50±0.9 µg/mL, which was probably due to a low percentage of beetroot juice. Figure A-2 represents the variation of AA, DHA and TAA among the different crops purchased from the different stores.

5.4.6 Total phenolic and radical scavenging activity

Variation in total phenolics content was evaluated by Folin-Ciocalteu reagents in the different vegetables juices and blends (**Figure 13A**) and is also represented for the different store as well (Figure A-3A). Results revealed that a significant difference ($P < 0.05$) was observed in the vegetable juices and their blends (B1–6). The total phenolic content in the leafy green vegetables was 968.81±15.2 µg GAE/mL for kale and 942.94±22.2 µg GAE/mL for arugula. These values were significantly higher than beetroot (569.18±15.7 µg GAE/mL) and watermelon (123.19±3.5 µg GAE/mL). The total phenolic content of individual vegetables also influenced the phenolic content among the different juices blends. Blend 2 had a significantly higher total phenolic content (515.11±7.8 µg GAE/mL), which may be due to highest content of kale and

arugula in this juice as compared with other blends. It was observed that, the overall total phenolic content decreased as the concentration of watermelon juice increased.

Evaluation of antioxidant activity is essential for the synergistic or antagonistic behavior of bio-active constituents and plays a dominant role in determining nutritional and health promoting functional qualities. The antioxidant activity of different vegetables juices and blends was established by *in-vitro* DPPH bioassay. The results

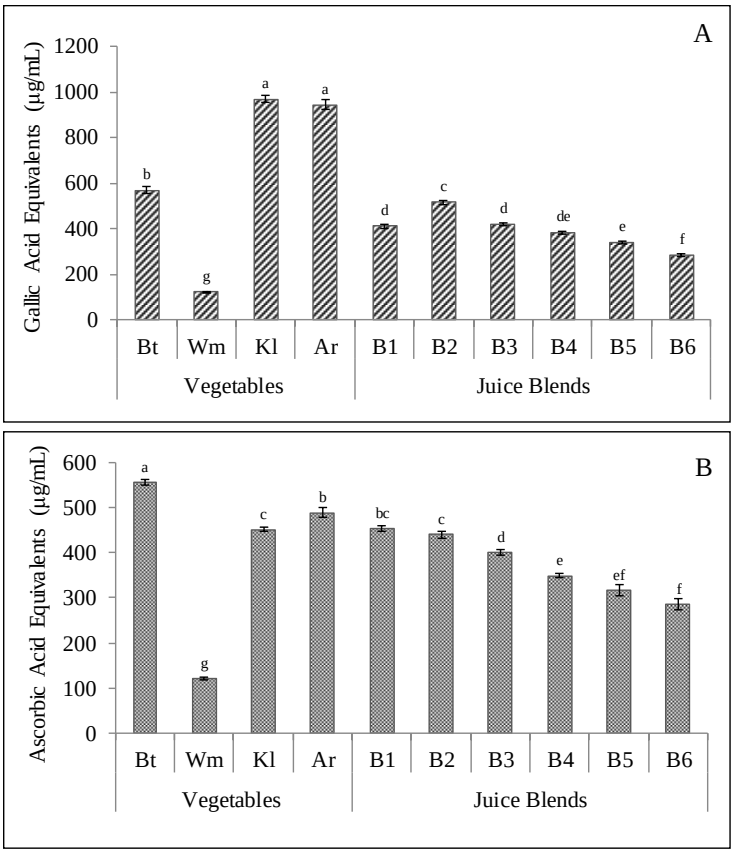

Figure 13. Total phenolic content and DPPH radical scavenging activity in beetroot, watermelon, kale, arugula, and blended juices. A) Total phenolic content, B) DPPH radical scavenging activity. Abbreviations represent Bt-beetroot, Wm-watermelon, Kl-kale, Ar-arugula, B1-blend 1, B2-blend 2, B3-blend 3, B4-blend 4, B5- blend 5, and B6-blend 6. Different letters denote a significant difference between values at $*p \leq 0.05$. Samples expressed as mean of 9 replicates (3 replications per store) ± SE.

demonstrate that beetroot juice had the highest DPPH radical scavenging activity which was found to be 554.95±6.1 µg AA/mL followed by kale (451.18±4.6 µg AA/mL) and arugula (488.76±10.6 µg AA/mL). Watermelon had the lowest scavenging activity which was 121.71±2.1 µg AA/mL (**Figure 13B**). The higher activity of beetroot and green leafy vegetables juices is due to the presence of betacyanin and polyphenols such as quercetin, kaempferol and their derivatives respectively.[33, 46, 79, 120] The blends B-1 and B-2 showed significantly higher activity 453.92±6.5 µg AA/mL and 440.36±7.6 µg AA/mL respectively, than all other blends. This can be attributed to a higher concentration beetroot juice. As the percentage of beetroot decreased in the blend juices, their antioxidant activity was also gradually reduced. Thus B-6 displayed the lowest antioxidant activity 285.72±12.8 µg AA/mL. Figure A-3B, represents the variation of antioxidant potential among the different stores.

5.4.7 Sugar content

The sugar content of the fresh juices and blends was evaluated by HPLC-RI and the results are tabulated in **Figure 14** and their variation among stores in Figure A-4. Beetroot juice had the highest content of sucrose (49.57±1.49 µg/mL); however no glucose and fructose were present. Our study is in agreement with published literature which reported that sucrose was the main sugar in beetroot.[28] Watermelon juice had the highest amount of fructose (31.01±0.53 µg/mL) compared to all the other juices. In kale, glucose (9.26±0.42 µg/mL) was higher than sucrose (7.62±0.54 µg/mL) and fructose (4.74±0.20 µg/mL). Previous studies have also reported higher levels of glucose and sucrose in kale.[145] Arugula had a higher glucose content (10.04±1.48 µg/mL) followed

by sucrose (5.73±0.60 µg/mL) and fructose (2.01±0.43 µg/mL). These results are in accordance to previous reports that show that glucose is the predominant sugar in arugula.[36]

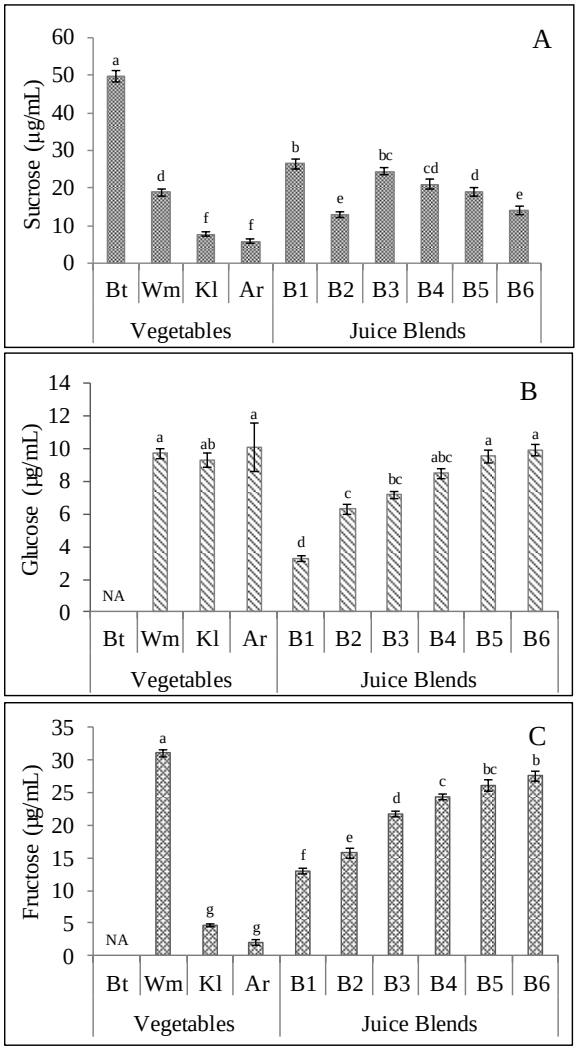

Figure 14. Sucrose (A), glucose (B), and fructose (C) in beetroot, watermelon, kale, arugula, and blended juices measured by high pressure liquid chromatography. Abbreviations represent Bt-beetroot, Wm-watermelon, Kl-kale, Ar-arugula, B1-blend 1, B2-blend 2, B3-blend 3, B4-blend 4, B5- blend 5, and B6-blend. Different letters denote a significant difference between values at $*p \leq 0.05$. Samples expressed as mean of 9 replicates (3 replications per store) ± SE.

5.4.8 Levels of amino acids

The amino acid profile for each vegetable juice and their juices blends were analyzed by HPLC-FLD and the results are tabulated in **Table 10.** The main amino acid in beetroot was found to be glutamine (2034.83±41.82 µg/mL) which was significantly higher than all the other juices. Published literature show that glutamine levels have been observed to change based upon fertilization conditions, and it acts as a precursor to betaxanthin (vulgaxanthin I) in beetroot.[25, 151] The amino acids tyrosine and citrulline were not detected in beetroot juice. For watermelon, the predominant amino acid was L-citrulline (1279.97±24.52 µg/mL). These results are consistent with reported literature that show that L-citrulline is the main amino acid in watermelon.[41, 42] Reported levels of L-citrulline in watermelon vary from 0.5–3.6 mg/g FW.[45]

Kale was found to have high levels of glutamine (1454.61±36.64 µg/mL), arginine (1088.00±29.90 µg/mL), and asparagine (1005.08±33.66 µg/mL). Variation in the predominant amino acids levels of kale has been reported.[50] However, arginine has been reported in high levels which are in accordance with our findings.[50, 54] For arugula juice the main amino acids present were glutamic acid (699.84±101.96 µg/mL), glutamine (675.76±54.58 µg/mL) and aspartic acid (492.62±43.56 µg/mL). These results are in agreement with previous report that showed glutamic acid and aspartic acid as the predominant amino acids, they also found that the levels of glutamine varied by cultivar.[152] For the juice blends the amino acid profile was mainly influence by the percentage of beetroot and watermelon in the blend. For blends that had a higher percentage of beetroot juice the main amino acid present in the blend was glutamine.

Table 10 The amino acid composition of beetroot, watermelon, kale, arugula and blended juices measured by high pressure liquid chromatography coupled with fluorescence detection.

Analyte	Beetroot	Watermelon	Kale	Arugula	Blend 1	Blend 2	Blend 3	Blend 4	Blend 5	Blend 6
Asp	305.45±7.30^{c}	111.47±3.53^{f}	593.01±17.32^{a}	492.62±43.56^{b}	183.44±8.75^{def}	249.39±10.18^{cd}	210.79±9.12^{de}	203.28±6.29^{de}	160.10±3.32^{ef}	165.92±8.58^{ef}
Glu	76.93±7.87^{c}	32.24±3.51^{c}	435.85±15.56^{b}	699.84±101.96^{a}	33.42±1.95^{c}	102.54±8.62^{c}	88.20±10.94^{c}	90.16±13.81^{c}	75.84±9.58^{c}	78.94±10.72^{c}
Asn	294.49±7.11^{b}	53.41±2.25^{f}	1005.08±33.66^{a}	202.44±21.21^{cd}	157.72±7.03^{de}	231.95±7.68^{c}	151.92±7.24^{de}	132.87±6.16^{e}	111.24±5.19^{ef}	104.50±3.39^{ef}
His	40.38±1.52^{f}	44.54±2.73^{f}	322.07±16.30^{a}	129.65±3.95^{a}	46.31±2.12^{f}	89.91±1.95^{c}	72.25±1.73^{cd}	53.92±2.77^{def}	67.42±1.28^{de}	49.93±2.79^{ef}
Ser	235.67±8.29^{c}	88.20±4.76^{f}	623.08±14.92^{a}	305.62±15.74^{b}	145.18±7.68^{de}	179.19±6.59^{d}	142.29±8.25^{de}	138.45±5.00^{e}	117.71±4.46^{ef}	108.85±4.75^{ef}
Gln	2034.83±41.82^{a}	297.57±20.58^{g}	1454.61±36.64^{b}	675.76±54.48^{f}	1295.05±35.89^{c}	1081.41±10.70^{d}	990.25±33.85^{de}	873.99±29.46^{e}	691.03±31.74^{f}	593.45±29.04^{f}
Cit	ND	1279.97±24.52^{a}	31.59±3.57^{e}	49.38±4.00^{e}	718.97±22.88^{d}	650.02±14.34^{d}	889.94±24.57^{c}	976.63±23.27^{bc}	936.96±27.42^{bc}	1001.16±35.70^{b}
Arg	25.41±1.20^{f}	460.23±9.32^{b}	1088.00±29.90^{a}	262.59±22.95^{de}	241.71±11.64^{e}	373.65±12.27^{c}	322.65±7.90^{cd}	343.25±8.47^{c}	323.62±6.69^{cd}	348.24±9.28^{c}
Gly	79.04±2.41^{b}	28.32±4.21^{c}	117.29±10.65^{a}	43.44±2.64^{c}	43.43±1.29^{c}	41.17±0.93^{c}	42.43±1.41^{c}	34.19±1.32^{c}	33.36±1.15^{c}	36.35±1.32^{c}
Thr	136.35±9.08^{c}	41.51±2.08^{e}	284.26±12.70^{a}	203.49±15.51^{b}	61.63±2.61^{de}	87.71±2.02^{d}	70.73±1.69^{de}	72.67±3.84^{de}	63.46±1.41^{de}	57.90±2.11^{de}
Ala	448.89±11.48^{a}	52.53±3.49^{f}	405.61±14.54^{b}	258.44±17.09^{c}	225.94±7.47^{c}	183.20±4.75^{d}	178.70±4.21^{d}	150.99±5.21^{d}	110.16±2.73^{e}	107.72±6.13^{e}
β-ala	90.20±2.18^{a}	31.24±1.00^{cd}	48.90±1.73^{b}	54.85±1.53^{b}	36.41±1.74^{cd}	35.54±1.69^{cd}	33.47±1.75^{cd}	38.85±1.94^{c}	38.11±0.70^{c}	30.47±1.16^{d}
Tyr	ND	23.68±1.32^{d}	119.15±4.29^{a}	100.89±5.68^{b}	51.31±5.42^{c}	37.20±3.42^{cd}	35.73±3.15^{cd}	16.79±0.32^{d}	16.38±0.19^{d}	16.19±0.45^{d}
Met	17.45±0.49^{e}	48.20±3.44^{a}	40.64±1.08^{ab}	41.66±1.59^{ab}	28.76±1.26^{d}	30.78±1.26^{cd}	34.32±2.03^{bcd}	36.29±1.99^{bcd}	34.53±2.18^{bcd}	38.27±2.53^{bc}
Val	117.49±4.63^{cd}	60.07±3.17^{e}	490.18±12.51^{a}	311.65±12.90^{b}	84.12±3.40^{de}	146.62±2.89^{c}	100.68±2.69^{d}	99.09±2.56^{d}	95.40±2.48^{d}	104.18±12.81^{d}
Trp	68.21±4.22^{de}	46.77±1.72^{e}	202.05±11.71^{a}	139.30±8.01^{b}	72.62±4.36^{cd}	94.95±6.11^{c}	96.77±2.96^{c}	60.95±2.56^{de}	53.17±2.61^{de}	52.71±2.88^{de}
Phe	32.35±2.46^{d}	106.13±3.21^{c}	393.36±15.39^{a}	309.30±14.19^{b}	61.57±2.82^{d}	121.18±4.63^{c}	96.39±3.23^{c}	105.97±3.43^{c}	100.87±2.46^{c}	105.78±3.72^{c}
Iso	186.01±4.75^{b}	97.51±2.87^{e}	274.57±7.80^{a}	176.04±8.75^{b}	137.39±5.01^{c}	139.49±3.65^{c}	120.69±3.71^{cd}	117.65±2.78^{cde}	106.86±2.11^{de}	107.03±4.46^{de}
Leu	135.96±4.96^{c}	56.18±3.49^{g}	217.34±6.31^{a}	156.87±8.05^{b}	92.74±4.48^{de}	96.42±2.99^{d}	79.75±2.76^{def}	73.01±2.11^{efg}	63.84±1.03^{fg}	66.06±2.74^{fg}
Orn	23.60±0.76^{bc}	18.18±0.88^{c}	28.33±1.36^{bc}	64.11±9.22^{a}	19.79±0.70^{bc}	31.50±2.68^{bc}	30.65±2.31^{bc}	34.34±3.09^{b}	17.57±0.52^{c}	27.56±2.50^{bc}
Lys	48.36±2.20^{de}	54.96±4.32^{de}	391.16±15.40^{a}	220.14±8.71^{b}	37.72±1.54^{e}	90.73±3.00^{c}	62.38±2.23^{de}	65.76±1.84^{cd}	58.83±1.22^{cd}	65.77±2.27^{cd}
Total	4366.61±82.34^{bc}	2989.77±66.77^{f}	8313.19±204.88^{a}	4803.90±188.47^{b}	3740.44±109.44^{de}	4067.85±72.54^{cd}	3820.14±82.95^{d}	3702.66±62.80^{de}	3265.54±61.22^{ef}	3247.98±105.15^{ef}

Abbreviations represent Asp-aspartic acid, Glu-glutamic acid, Asn-asparagine, His-histidine, Ser-serine, Gln-glutamine, Cit-citrulline, Arg-arginine, Gly-glycine, Thr-threonine, Ala-alanine, β-ala-β-alanine, Tyr-tyrosine, Met-methionine, Val-valine, Trp-tryptophan, Phe-phenylalanine, Iso-isoleucine, Leu-leucine, Orn-ornithine, and Lys-lysine. Different letters denote a significant difference between values at *$p \leq 0.05$. Samples expressed as mean of 9 replicates (3 replications per store) ± SE. ND-not detected.

For B-1, the level of glutamine (1295.05±35.89 µg/mL) was significantly higher than all other blends. For B-5 (691.03±31.74 µg/mL) and B-6 (593.45±29.04 µg/mL) with the lowest percentage of beetroot, glutamine was significantly lower. L-citrulline, the major amino acid present in watermelon, was observed to increase as the percentage of watermelon increased. In B-6 (80% Wm) the citrulline content (1001.16±35.70 µg/mL) was significantly higher than the other juice blends. It was also observed than B-1 (718.97±22.88 µg/mL) and B-2 (650.02±14.34 µg/mL) with the lowest watermelon juice content were significantly lower among all blends. Figure A-5 displays the scatter plot for glutamine and citrulline content in the juice blends. For the total amino acid content, levels did not vary drastically; B-2 with the highest amount of leafy greens had the highest amount (4067.85±72.54 µg/mL). The overall range for total amino acids in the juice blends was from 4067.85±72.54 to 3247.98±105.15 µg/mL. For the vegetable juices kale had significantly higher amino acid content (8313.19±204.88 µg/mL) followed by arugula (4803.90±188.47 µg/mL), beetroot (4366.61±82.34 µg/mL), and watermelon (2989.77±66.77 µg/mL).

5.4.9 Nitrate and L-citrulline content of freeze-dried juice

On the HEB store juices a separate study was performed to analyze the nitrate content in the freeze-dried juice samples. Literature shows that nitrate plays an important role in reduction of blood pressure and improvement of vascular compliance. [8] Thus, nitrate is commonly used as a pharmacological agent for the treatment of cardiovascular disorders.[153] Recently, due to an emerging understanding of the involvement of nitrate in various physiological processes, nitrate supplementation has

gained interest, especially for cardiovascular function and enhancement of sports performance.[8, 154] Previously reported literature showed that the consumption of nitrate-rich supplements improved sports performance.[59, 154] Most studies used nitrate doses of 300-600mg, which were found to show potential sports performance benefits as compared to lowers dosages.[155, 156] **Figure 15A** represents the nitrate content in freeze-dried juices sample from different vegetables and juices blends. Results demonstrated that there is a significant difference between the nitrate content in the freeze-dried juices. Beetroot freeze-dried juice showed the highest nitrate content, as expected. Among the juice blends, B-1 had the highest nitrate content, due to a higher percentage (50%) of beetroot.

Due to the importance of L-citrulline in the endogenous production of nitric oxide via the NOS dependent pathway, the content in the preliminary freeze-dried samples was evaluated. Studies show a dose-dependent response to oral L-citrulline supplementation that can raise plasma L-arginine concentrations and augment NO-dependent signaling.[157] Supplementation with 6 g/day of L-citrulline over a seven day period showed improved endurance exercise performance, enhanced speed $\dot{V}o_2$ kinetics and reduced blood pressure.[72] The results are displayed in **Figure 15B**. Figure A-6 represents the scatter plot matrix for nitrate and L-citrulline for the six freeze-dried juice blends. Watermelon had significantly higher levels of L-citrulline (7.59±0.14 mg/g) than all other vegetables and blends. Levels were followed by B-6 (4.80±0.08 mg/g), B-5 (4.26±0.20 mg/g), and B-4 (4.24±0.12 mg/g) in which all had high percentages of watermelon juice. These result are in accordance with what was expected, since

watermelon has the highest reported levels of L-citrulline among all vegetables and fruits.[45, 109] Overall, as the percentage of watermelon juice decreased, the content of L-citrulline also decreased in the freeze-dried juice blends.

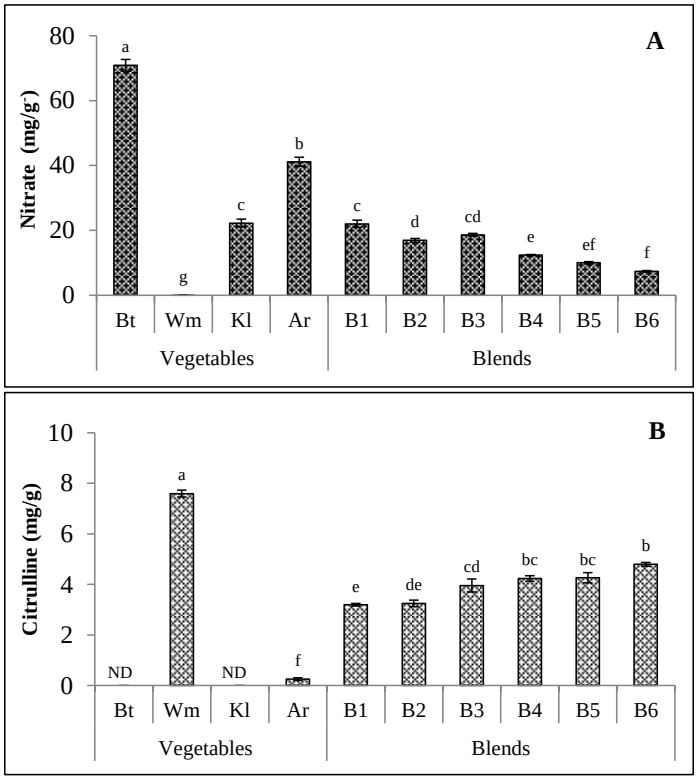

Figure 15. The nitrate (A) and L-citrulline (B) content for the preliminary freeze-drying experiment for beetroot, watermelon, kale, arugula, and blended juices measured by high pressure liquid chromatography. Abbreviations represent Bt-beetroot, Wm-watermelon, Kl-kale, Ar-arugula, B1-blend 1, B2-blend 2, B3-blend 3, B4-blend 4, B5- blend 5, and B6-blend 6. Different letters denote a significant difference between values at $*p \leq 0.05$ Samples expressed as mean of 3 replications ± SE. ND-not detected.

5.5 Conclusion

The results demonstrated that the blending of juices from different vegetables (watermelon, beetroot, kale, and arugula) significantly affected the phytochemical

profiles of the juices. The nitrate content in the juices was mainly dependent upon the beetroot juice concentration, while ascorbic acid content mainly depended on the green leafy vegetables (kale and arugula).Watermelon significantly affected the L-citrulline content in the juice blends. The technique of freeze drying may also play an important role in maintaining the nitrate and L-citrulline content in the lyophilized juice powers which can be used for the preparation of functional supplements for potentially enhancing nitric oxide precursors. Moreover, blending of juices also can also help in the development of novel functional juices by enhancing the nutritional profile of the specific juices, which are devoid of specific phytochemicals.

CHAPTER VI

SUMMARY AND CONCLUSIONS

Amino acids are involved in numerous biological processes, including protein synthesis, cell signaling, cellular metabolism, and immune response. For example, L-citrulline is a non-essential amino acid engaged in interorgan metabolism.[61, 65] It is also involved in the production of nitric oxide, a signaling molecule and potent vasodilator. Few vegetables are rich sources of L-citrulline.[45] Analysis of L-citrulline and other free amino acids in juices from different vegetables is important for the determination of quality and health benefits. In the present study, an optimized rapid, sensitive and reproducible analytical method was developed for the separation and quantification of free amino acids, including L-citrulline, from various vegetable juices and commercial juice samples. The optimal conditions for analysis of amino acids were obtained with 20 mM sodium acetate (solvent A) and water with organic modifiers acetonitrile and methanol. Other parameters such as pH, column temperature, precision, limit of detection and limit of quantification, were also evaluated. The developed method was applied for the analysis of amino acids from watermelon, cucumber, celery, calabaza squash, zucchini squash, yellow squash, and commercial juice samples.

Nitrate and polyphenols from the diet may enhance the production and bioavailability of nitric oxide, a radical signaling molecule involved in cardiovascular function. The stability of nitrate and total phenolics in beetroot and arugula juices was measured over a one-month storage period at different temperatures. The levels of nitrate were measured by reversed phase HPLC and the results demonstrated that storage

temperature and period had significant effects on the levels of nitrate and nitrite. At 25°C, the reduction of nitrate started after one day of storage, which caused an increase in nitrite content. At 4°C, nitrate was stable for a longer time without any noticeable nitrite levels. Storing samples in frozen conditions prevented the loss of nitrate due to conversion to nitrite in the juices of both vegetables during a one-month storage period. Refrigeration may help prevent a decrease in nitrate levels in raw vegetable juices over a short time frame. For long-term storage however, freezing is required to prevent a decrease in nitrate content. The stability of betacyanins was also influence by the storage conditions. An increase in the antioxidant activity of arugula juice was observed due to the breakdown of flavonoids glucosides to their aglycone moieties or lower phenolic compounds, which was confirmed by UPLC-HR-QTOF-MS analysis. These results may lead to the investigation of optimizing conditions that retain nitrate and stimulate the breakdown of flavonoids glucosides to their alcyone moieties in a safe manner for functional foods.

Dietary nitrate and L-citrulline are precursors to nitric oxide, a potent vasodilator that benefits cardiovascular health and sports performance. Certain vegetables contain these compounds and combining these vegetables into juice blends may lead to a natural functional beverage with beneficial levels of nitric oxide and its precursors. In the present study, juices of the selected vegetables (watermelon, beetroot, kale, and arugula) were mixed to determine optimal juice blends to maximize the levels of nitric oxide precursors such as nitrate and L-citrulline. The results demonstrated that nitrate content in the juices was mainly dependent upon the beetroot juice concentration while ascorbic

acid content depended on the green leafy vegetables, kale and arugula. Beetroot juice had the highest level of nitrate followed by kale and arugula. For the blends, watermelon significantly affected the L-citrulline content in the juice blends. Watermelon juice and blend B-6 (with a high percentage of watermelon) were found to have the highest levels of L-citrulline. The juices were also freeze-dried and then evaluated for their nitrate and L-citrulline content. The technique of freeze drying may play an essential role in maintaining the nitrate and L-citrulline content in the lyophilized juice powers. These samples may be useful for the preparation of functional supplements that could increase the levels of nitric oxide precursors. In conclusion, the blending of vegetable juices can increase the levels of bioactive compounds and enhance the nitrate and L-citrulline content which may lead to improved functional properties.

REFERENCES

1. J. L. Slavin and B. Lloyd, *Adv. Nutr. (BETHESDA)*, 2012, **3**, 506-516.
2. R. H. Liu, *Adv. Nutr. (BETHESDA)*, 2013, **4**, 384S-392S.
3. R. H. Liu, *Am. J. Clin. Nutr.*, 2003, **78**, 517S-520S.
4. D. S. Seigler, *Plant secondary metabolism*, Springer Science & Business Media, 2012.
5. S. McGuire, *Adv. Nutr. (BETHESDA)*, 2013, **4**, 665-666.
6. D. J. Lamport, C. Saunders, L. T. Butler and J. P. Spencer, *Nutr. Rev.*, 2014, **72**, 774-789.
7. P. C. Wootton-Beard and L. Ryan, *Food Res. Int.*, 2011, **44**, 3135-3148.
8. S. Lidder and A. J. Webb, *Br. J. Clin. Pharmacol.*, 2013, **75**, 677-696.
9. D. J. Lamport, C. Saunders, L. T. Butler and J. P. Spencer, *Nutrition reviews*, 2014, **72**, 774-789.
10. R. L. Bhardwaj and S. Pandey, *Crit. Rev. Food Sci. Nutr.*, 2011, **51**, 563-570.
11. P. C. Wootton-Beard and L. Ryan, *J. Funct. Foods*, 2011, **3**, 329-334.
12. M. P. Tarazona-Díaz, F. Alacid, M. Carrasco, I. Martínez and E. Aguayo, *J. Agric. Food. Chem.*, 2013, **61**, 7522-7528.
13. L. Deldicque and M. Francaux, *Curr. Opin. Clin. Nutr. Metab. Care*, 2008, **11**, 774-781.
14. W. Aoi, Y. Naito and T. Yoshikawa, *Nutr. J.*, 2006, **5**, 15.
15. G. Y. Wu, *Functional Amino Acids in Growth, Reproduction, and Health*.
16. L. Galland, 2005.

17. K. Iqbal, A. Khan and M. M. A. K. Khattak, *Pakistan J. of Nutr.*, 2004, **3**, 5-13.

18. B. R. Flam, D. C. Eichler and L. P. Solomonson, *Nitric Oxide*, 2007, **17**, 115-121.

19. K. E. Lansley, P. G. Winyard, S. J. Bailey, A. Vanhatalo, D. P. Wilkerson, J. R. Blackwell, M. Gilchrist, N. Benjamin and A. M. Jones, *Med. Sci. Sports Exerc.*, 2011, **43**, 1125-1131.

20. M. J. Ormsbee, J. Lox and P. J. Arciero, *Nutr Diet Suppl.*, 2013, **5**, 27-35.

21. A. Jones, *Sports Med.*, 2014, **44**, 35-45.

22. G. K. Jayaprakasha, K. N. Chidambara Murthy and B. S. Patil, *Food Chem.*, 2011, **127**, 240-248.

23. F. Zeng, J. Ou, Y. Huang, Q. Li, G. Xu, Z. Liu and S. Yang, *Food Anal. Methods*, 2015, **8**, 428-437.

24. A. Acquaviva, L. Romero, C. Castells, G. Ramis-Ramos and J. M. Herrero-Martinez, *Anal. Methods* 2014, **6**, 5830-5837.

25. B. Nemzer, Z. Pietrzkowski, A. Spórna, P. Stalica, W. Thresher, T. Michałowski and S. Wybraniec, *Food Chem.*, 2011, **127**, 42-53.

26. T. K. Koley, C. Kaur, S. Nagal, S. Walia and S. Jaggi, *Arab. J. Chem.* , 2016, **9**, S1044-S1052.

27. J. K. Collins, G. Wu, P. Perkins-Veazie, K. Spears, P. L. Claypool, R. A. Baker and B. A. Clevidence, *Nutrition*, 2007, **23**, 261-266.

28. J. Wruss, G. Waldenberger, S. Huemer, P. Uygun, P. Lanzerstorfer, U. Müller, O. Höglinger and J. Weghuber, *J. Food. Comp. Anal.*, 2015, **42**, 46-55.

29. T. Clifford, G. Howatson, D. J. West and E. J. Stevenson, *Nutrients*, 2015, **7**, 2801-2822.

30. A. J. Webb, N. Patel, S. Loukogeorgakis, M. Okorie, Z. Aboud, S. Misra, R. Rashid, P. Miall, J. Deanfield and N. Benjamin, *Hypertension*, 2008, **51**, 784-790.

31. L. Bell and C. Wagstaff, *J. Agric. Food. Chem.*, 2014, **62**, 4481-4492.

32. J. Jin, O. A. Koroleva, T. Gibson, J. Swanston, J. Magan, Y. Zhang, I. R. Rowland and C. Wagstaff, *J. Agric. Food. Chem.*, 2009, **57**, 5227-5234.

33. B. H. Abbasi, J. Ali, M. Ali, M. Zia, S. A. Bokhari and M. A. Khan, *Toxicol. Ind. Health*, 2016, **32**, 98-105.

34. E. Fuentes, M. Alarcón, M. Fuentes, G. Carrasco and I. Palomo, *Nutrients*, 2014, **6**, 5839-5852.

35. M. Cavaiuolo and A. Ferrante, *Nutrients*, 2014, **6**, 1519-1538.

36. M. Villatoro-Pulido, F. Priego-Capote, B. Álvarez-Sánchez, S. Saha, M. Philo, S. Obregón-Cano, D. Haro-Bailón, R. Font and D. Río-Celestino, *J. Sci. Food Agric.*, 2013, **93**, 3809-3819.

37. S.-J. Kim and G. Ishii, *J. Sci. Food Agric.*, 2007, **87**, 966-973.

38. M. H. Hetta, A. I. Owis, P. S. Haddad and H. M. Eid, *Pharm. Biol.*, 2017, **55**, 810-818.

39. J. O. Lundberg, E. Weitzberg, J. A. Cole and N. Benjamin, *Nat. Rev. Microbiol.*, 2004, **2**, 593-602.

40. J. O. Lundberg, M. Carlström, F. J. Larsen and E. Weitzberg, *Cardiovasc. Res.*, 2011, **89**, 525-532.

41. P. Perkins-Veazie, A. Davis and J. K. Collins, *Israel Journal of Plant Sciences*, 2012, **60**, 395-402.

42. I. Tlili, C. Hdider, M. S. Lenucci, I. Riadh, H. Jebari and G. Dalessandro, *J. Food. Comp. Anal.*, 2011, **24**, 307-314.

43. M. P. Tarazona-Díaz, J. Viegas, M. Moldao-Martins and E. Aguayo, *J. Sci. Food Agric.*, 2011, **91**, 805-812.

44. A. J. Edwards, B. T. Vinyard, E. R. Wiley, E. D. Brown, J. K. Collins, P. Perkins-Veazie, R. A. Baker and B. A. Clevidence, *J. Nutr.*, 2003, **133**, 1043-1050.

45. A. M. Rimando and P. M. Perkins-Veazie, *J. Chromatogr. A*, 2005, **1078**, 196-200.

46. M. Zietz, A. Weckmüller, S. Schmidt, S. Rohn, M. Schreiner, A. Krumbein and L. W. Kroh, *J. Agric. Food. Chem.*, 2010, **58**, 2123-2130.

47. H. Olsen, K. Aaby and G. I. A. Borge, *J. Agric. Food. Chem.*, 2009, **57**, 2816-2825.

48. P. Velasco, M. E. Cartea, C. González, M. Vilar and A. Ordás, *J. Agric. Food. Chem.*, 2007, **55**, 955-962.

49. S. Schmidt, M. Zietz, M. Schreiner, S. Rohn, L. W. Kroh and A. Krumbein, *Rapid Commun. Mass Sp.*, 2010, **24**, 2009-2022.

50. Z. Lisiewska, W. Kmiecik and A. Korus, *Food Chem.*, 2008, **108**, 642-648.

51. A. Korus, *J. Sci. Food Agric.*, 2012, **92**, 618-625.

52. S. F. Hagen, G. I. A. Borge, K. A. Solhaug and G. B. Bengtsson, *Postharvest Biol. Technol.*, 2009, **51**, 36-42.

53. W. H. Eppendorfer and S. W. Bille, *J. Sci. Food Agric.*, 1996, **71**, 458.

54. F. A. Ayaz, R. H. Glew, M. Millson, H. Huang, L. Chuang, C. Sanz and S. Hayırlıoglu-Ayaz, *Food Chem.*, 2006, **96**, 572-579.

55. T. Y. K. Chan, *Toxicol. Lett.* , 2011, **200**, 107-108.

56. M. Govoni, E. A. Jansson, E. Weitzberg and J. O. Lundberg, *Nitric Oxide*, 2008, **19**, 333-337.

57. K. E. Lansley, P. G. Winyard, S. J. Bailey, A. Vanhatalo, D. P. Wilkerson, J. R. Blackwell, M. Gilchrist, N. Benjamin and A. M. Jones, *Med Sci Sports Exerc*, 2011, **43**, 1125-1131.

58. K. E. Lansley, P. G. Winyard, J. Fulford, A. Vanhatalo, S. J. Bailey, J. R. Blackwell, F. J. DiMenna, M. Gilchrist, N. Benjamin and A. M. Jones, *J. Appl. Physiol.*, 2011, **110**, 591-600.

59. H. Bond, L. Morton and A. J. Braakhuis, *Int. J. Sport Nutr. Exerc. Metab.*, 2012, **22**, 251-256.

60. G. Wu, *Amino acids : biochemistry and nutrition*, Boca Raton : Taylor & Francis, [2013], 2013.

61. G. Wu, Z. Wu, Z. Dai, Y. Yang, W. Wang, C. Liu, B. Wang, J. Wang and Y. Yin, *Amino Acids*, 2013, **44**, 1107-1113.

62. G. Wu, F. W. Bazer, T. A. Davis, S. W. Kim, P. Li, J. Marc Rhoads, M. Carey Satterfield, S. B. Smith, T. E. Spencer and Y. Yin, *Amino Acids*, 2009.

63. E. Curis, I. Nicolis, C. Moinard, S. Osowska, N. Zerrouk, S. Bénazeth and L. Cynober, *Amino Acids*, 2005, **29**, 177-205.

64. M. Wada, *Biochem. Zeits*, 1930, **224**, 420-429.

65. S. Bahri, N. Zerrouk, C. Aussel, C. Moinard, P. Crenn, E. Curis, J.-C. Chaumeil, L. Cynober and S. Sfar, *Nutrition*, 2013, **29**, 479-484.

66. H. G. Windmueller and A. E. Spaeth, *J. Biol. Chem.*, 1974, **249**, 5070-5079.

67. Z. Dai, Z. Wu, Y. Yang, J. Wang, M. C. Satterfield, C. J. Meininger, F. W. Bazer and G. Wu, *BioFactors*, 2013, **39**, 383-391.

68. G. Wu and S. M. Morris, *Biochem. J*, 1998, **336**, 1-17.

69. S. M. Morris, Jr., *J. Nutr.*, 2004, **134**, 2743S-2747S; discussion 2765S-2767S.

70. I.-Y. Kim, S. E. Schutzler, A. Schrader, H. J. Spencer, G. Azhar, N. E. P. Deutz and R. R. Wolfe, *Am. J. Physiol. Endocrinol. Metab.*, 2015, **309**, E915-E924.

71. P. T. Cutrufello, S. J. Gadomski and G. S. Zavorsky, *J. Sports Sci.*, 2015, **33**, 1459-1466.

72. S. J. Bailey, J. R. Blackwell, T. Lord, A. Vanhatalo, P. G. Winyard and A. M. Jones, *J. Appl. Physiol.*, 2015, **119**, 385-395.

73. S.-J. Chien, K.-M. Lin, H.-C. Kuo, C.-F. Huang, Y.-J. Lin, L.-T. Huang and Y.-L. Tain, *Transl. Res.*, 2014, **163**, 43-52.

74. T. Tamme, M. Reinik, T. Püssa, M. Roasto, K. Meremäe and A. Kiis, *Food Addit. Contam.*, 2010, **27**, 487-495.

75. S. Prasad and A. A. Chetty, *Food Chem.*, 2008, **106**, 772-780.

76. Q.-H. Wang, L.-J. Yu, Y. Liu, L. Lin, R.-g. Lu, J.-p. Zhu, L. He and Z.-L. Lu, *Talanta*, 2017, **165**, 709-720.

77. F. Pasini, V. Verardo, M. F. Caboni and L. F. D'Antuono, *Food Chem.*, 2012, **133**, 1025-1033.

78. J. Hsu, J. Arcot and N. A. Lee, *Food Chem.*, 2009, **115**, 334-339.

79. L. Bell, M. J. Oruna-Concha and C. Wagstaff, *Food Chem.*, 2015, **172**, 852-861.

80. A. Martínez-Sánchez, R. Llorach, M. I. Gil and F. Ferreres, *J. Agric. Food Chem.*, 2007, **55**, 1356-1363.

81. R. Mandrioli, L. Mercolini and M. A. Raggi, *Anal. Bioanal. Chem.*, 2013, **405**, 7941-7956.

82. P. Hernández-Orte, M. Ibarz, J. Cacho and V. Ferreira, *Chromatographia*, 2003, **58**, 29-35.

83. A. Fabiani, A. Versari, G. Parpinello, M. Castellari and S. Galassi, *J. Chromatogr. Sci.*, 2002, **40**, 14-18.

84. R. Gheshlaghi, J. M. Scharer, M. Moo-Young and P. L. Douglas, *Anal. Biochem.*, 2008, **383**, 93-102.

85. A. Le, A. Ng, T. Kwan, K. Cusmano-Ozog and T. M. Cowan, *J. Chromatogr. B*, 2014, **944**, 166-174.

86. L. Wang, R. Xu, B. Hu, W. Li, Y. Sun, Y. Tu and X. Zeng, *Food Chem.*, 2010, **123**, 1259-1266.

87. Z. Dai, Z. Wu, S. Jia and G. Wu, *J. Chromatogr. B*, 2014, **964**, 116-127.

88. W. Li, M. Hou, Y. Cao, H. Song, T. Shi, X. Gao and D. Wang, *Food Anal. Methods*, 2012, **5**, 62-68.

89. M. Zhao, Y. Ma, L.-l. Dai, D.-l. Zhang, J.-h. Li, W.-x. Yuan, Y.-l. Li and H.-j. Zhou, *Food Anal. Methods*, 2013, **6**, 69-75.

90. G. Li, Y. Cui, J. You, X. Zhao, Z. Sun, L. Xia, Y. Suo and X. Wang, *Amino Acids*, 2011, **40**, 1185-1193.

91. L. E. Jayachandran, S. Chakraborty and P. S. Rao, *Innov. Food Sci. & Emerg. Technol.*, 2015, **28**, 1-9.

92. S. K. Jain and D. Khurdiya, *Plant Foods Hum. Nutr.* , 2004, **59**, 63-66.

93. M. J. Rodríguez-Roque, B. de Ancos, C. Sánchez-Moreno, M. P. Cano, P. Elez-Martínez and O. Martín-Belloso, *J. Funct. Foods*, 2015, **14**, 33-43.

94. A. Jumde, R. Shukla and Gousoddin, *Int. J. Sci. Eng. Technol.*, 2015, **3**, 960-964.

95. P. Khandpur and P. R. Gogate, *Ultrason. Sonochem.*, 2015, **27**, 125-136.

96. Q. Dai, A. R. Borenstein, Y. Wu, J. C. Jackson and E. B. Larson, *Am. J. Med.*, 2006, **119**, 751-759.

97. R. Clemens, A. Drewnowski, M. G. Ferruzzi, C. D. Toner and D. Welland, *Adv. Nutr. (BETHESDA)*, 2015, **6**, 236S-243S.

98. B. M. Silva, S. Casal, P. B. Andrade, R. M. Seabra, M. B. P. Oliveira and M. A. Ferreira, *J. Agric. Food. Chem.*, 2004, **52**, 1201-1206.

99. M. Asadpoor, M. Ansarin and M. Nemati, *Adv. Pharm. Bull.*, 2014, **4**, 359.

100. G. Wu, *Amino Acids*, 2009, **37**, 1-17.

101. M. Forte, V. Conti, A. Damato, M. Ambrosio, A. A. Puca, S. Sciarretta, G. Frati, C. Vecchione and A. Carrizzo, *Oxid. Med. Cell Longev.*, 2016, **2016**.

102. I. Krause, A. Bockhardt, H. Neckermann, T. Henle and H. Klostermeyer, *J. Chromatogr. A*, 1995, **715**, 67-79.

103. M. Castellanos, C. Van Van Eendenburg, C. Gubern and J. M. Sanchez, *J. Chromatogr. B*, 2016, **1029**, 137-144.

104. I. Pérez-Neri, E. Castro, S. Montes, M.-C. Boll, J. Barges-Coll, J. L. Soto-Hernández and C. Ríos, *J. Chromatogr. B*, 2007, **851**, 250-256.

105. G. Wu and C. J. Meininger, in *Methods Enzymol.*, Academic Press, 2008, vol. Volume 440, pp. 177-189.

106. H. Brückner and T. Westhauser, *Amino Acids*, 2003, **24**, 43-55.

107. W. W. Fish, *Food Nutr. Sci.*, 2012, **3**, 863.

108. A. R. Davis, C. L. Webber, W. W. Fish, T. C. Wehner, S. King and P. Perkins-Veazie, *HortScience*, 2011, **46**, 1572-1575.

109. G. Soteriou, M. Kyriacou, A. Siomos and D. Gerasopoulos, *Food Chem.*, 2014, **165**, 282-289.

110. Y. Liu, H.-B. Chen, Y.-Y. Zhao, B. Wang, Q.-Y. Zhang, L. Zhang and P. Tu, *J. Agric. Food. Chem.*, 2006, **54**, 6634-6639.

111. Y. C. Luiking, M. P. Engelen and N. E. Deutz, *Curr. Opin. Clin. Nutr. Metab. Care*, 2010, **13**, 97.

112. J. Duarte, V. Francisco and F. Perez-Vizcaino, *Food Funct.*, 2014, **5**, 1653-1668.

113. T. Michel and P. M. Vanhoutte, *Pflüg. Arch. Eu.r J. Phy.*, 2010, **459**, 807-816.

114. C. P. Bondonno, K. D. Croft, N. Ward, M. J. Considine and J. M. Hodgson, *Nutr. Res.*, 2015, **73**, 216-235.

115. Y. Aguilera, M. A. Martin-Cabrejas and E. G. de Mejia, *Phytochem. Rev.*, 2016, **15**, 405-423.

116. N. G. Hord, Y. Tang and N. S. Bryan, *Am. J. Clin. Nutr.*, 2009, **90**, 1-10.

117. E. Weitzberg and J. O. Lundberg, *Annu. Rev. Nutr.*, 2013, **33**, 129-159.

118. J. J. Doel, N. Benjamin, M. P. Hector, M. Rogers and R. P. Allaker, *Eur. J. Oral Sci.*, 2005, **113**, 14-19.

119. H. Si, R. P. Wyeth and D. Liu, *Eur. J. Clin. Nutr.*, 2014, **53**, 269-275.

120. T. S. Kujala, J. M. Loponen, K. D. Klika and K. Pihlaja, *J. Agric. Food. Chem.*, 2000, **48**, 5338-5342.

121. T. S. Kujala, M. S. Vienola, K. D. Klika, J. M. Loponen and K. Pihlaja, *Eur. Food Res. Technol.*, 2002, **214**, 505-510.

122. V. G. Georgiev, J. Weber, E.-M. Kneschke, P. N. Denev, T. Bley and A. I. Pavlov, *Plant Foods Hum. Nutr.*, 2010, **65**, 105-111.

123. K. M. Herbach, C. Maier, F. C. Stintzing and R. Carle, *Eur. Food Res. Technol.*, 2007, **224**, 649-658.

124. K. M. Herbach, F. C. Stintzing and R. Carle, *J. Food Sci.*, 2006, **71**.

125. H. M. Azeredo, A. N. Santos, A. C. Souza, K. C. Mendes and M. I. R. Andrade, *Am. J. Food Technol.*, 2007, **2**, 307-312.

126. F. C. Stintzing and R. Carle, *Trends Food Sci. Technol.*, 2007, **18**, 514-525.

127. M. Biesaga, *J. Chromatogr. A*, 2011, **1218**, 2505-2512.

128. E. Corradini, P. Foglia, P. Giansanti, R. Gubbiotti, R. Samperi and A. Laganà, *Nat. Prod. Res.*, 2011, **25**, 469-495.

129. M. Plaza, T. Pozzo, J. Liu, K. Z. Gulshan Ara, C. Turner and E. Nordberg Karlsson, *J. Agric. Food. Chem.*, 2014, **62**, 3321-3333.

130. A. A. van der Sluis, M. Dekker and M. A. J. S. van Boekel, *J. Agric. Food. Chem.*, 2005, **53**, 1073-1080.

131. G. Jayaprakasha and B. S. Patil, *Food Chem.*, 2007, **101**, 410-418.

132. G. K. Jayaprakasha, B. Girennavar and B. S. Patil, *LWT-Food Sci. Technol.*, 2008, **41**, 376-384.

133. J. C. Chung, S. S. Chou and D. F. Hwang, *Food Addit. Contam.*, 2004, **21**, 317-322.

134. W. Kmiecik, Z. Lisiewska and J. Słupski, *Food Chem.*, 2004, **86**, 105-111.

135. A. Gliszczyńska-Świgło, H. Szymusiak and P. Malinowska, *Food Addit. Contam.*, 2006, **23**, 1079-1087.

136. I. Klimczak, M. Małecka, M. Szlachta and A. Gliszczyńska-Świgło, *J. Food Comp. Anal.*, 2007, **20**, 313-322.

137. M. Anese, L. Manzocco, M. C. Nicoli and C. R. Lerici, *J. Sci. Food Agric.*, 1999, **79**, 750-754.

138. G. K. Jayaprakasha, L. Jaganmohan Rao and K. K. Sakariah, *Biorg. Med. Chem.*, 2004, **12**, 5141-5146.

139. H. P. Hendrickson, A. D. Kaufman and C. E. Lunte, *J. Pharm. Biomed. Anal.*, 1994, **12**, 325-334.

140. J. Xiao, *Crit. Rev. Food Sci. Nutr.*, 2017, **57**, 1874-1905.

141. M. Leenders, H. C. Boshuizen, P. Ferrari, P. D. Siersema, K. Overvad, A. Tjønneland, A. Olsen, M.-C. Boutron-Ruault, L. Dossus and L. Dartois, *Eur. J. Epidemiol.*, 2014, **29**, 639-652.

142. U. USDA, *Washington (DC): USDA*, 2015.

143. A. Abdel-Salam, *Am. J. Food Technol.*, 2010, **5**, 86-99.

144. R. Clemens, A. Drewnowski, M. G. Ferruzzi, C. D. Toner and D. Welland, *Advances in Nutrition: An International Review Journal*, 2015, **6**, 236S-243S.

145. S. Noichinda, K. Bodhipadma, C. Mahamontri, T. Narongruk and S. Ketsa, *Postharvest Biol. Technol.*, 2007, **44**, 312-315.

146. S.-S. Chou, J. Chung and D. Hwang, *J. Food Drug Anal.*, 2003, **11**, 233-238.

147. K. K. Chebrolu, G. Jayaprakasha, K. S. Yoo, J. L. Jifon and B. S. Patil, *LWT-Food Sci.Technol.*, 2012, **47**, 443-449.

148. K. S. Yoo, E. J. Lee and B. S. Patil, *J. Food Sci.*, 2012, **77**, C216-C221.

149. V. Khandare, S. Walia, M. Singh and C. Kaur, *Food Bioprod. Process.*, 2011, **89**, 482-486.

150. E. Colonna, Y. Rouphael, G. Barbieri and S. De Pascale, *Food Chem.*, 2016, **199**, 702-710.

151. H. Böhm and G. Mäck, *Phytochemistry*, 2004, **65**, 1361-1368.

152. L. Bell, L. Methven, A. Signore, M. J. Oruna-Concha and C. Wagstaff, *Food Chem.*, 2017, **218**, 181-191.

153. A. R. Butler and M. Feelisch, *Circulation*, 2008, **117**, 2151-2159.

154. S. J. Bailey, P. Winyard, A. Vanhatalo, J. R. Blackwell, F. J. DiMenna, D. P. Wilkerson, J. Tarr, N. Benjamin and A. M. Jones, *J. Appl. Physiol.*, 2009, **107**, 1144-1155.

155. A. Naderi, E. P. de Oliveira, T. N. Ziegenfuss and M. E. Willems, *J. Exerc. Nutrition Biochem.*, 2016, **20**, 1.

156. M. W. Hoon, N. A. Johnson, P. G. Chapman and L. M. Burke, *Int. J. Sport Nutr. Exerc. Metab.*, 2013, **23**, 522-532.

157. E. Schwedhelm, R. Maas, R. Freese, D. Jung, Z. Lukacs, A. Jambrecina, W. Spickler, F. Schulze and R. H. Böger, *Br. J. Clin. Pharmacol.*, 2008, **65**, 51-59.

APPENDIX

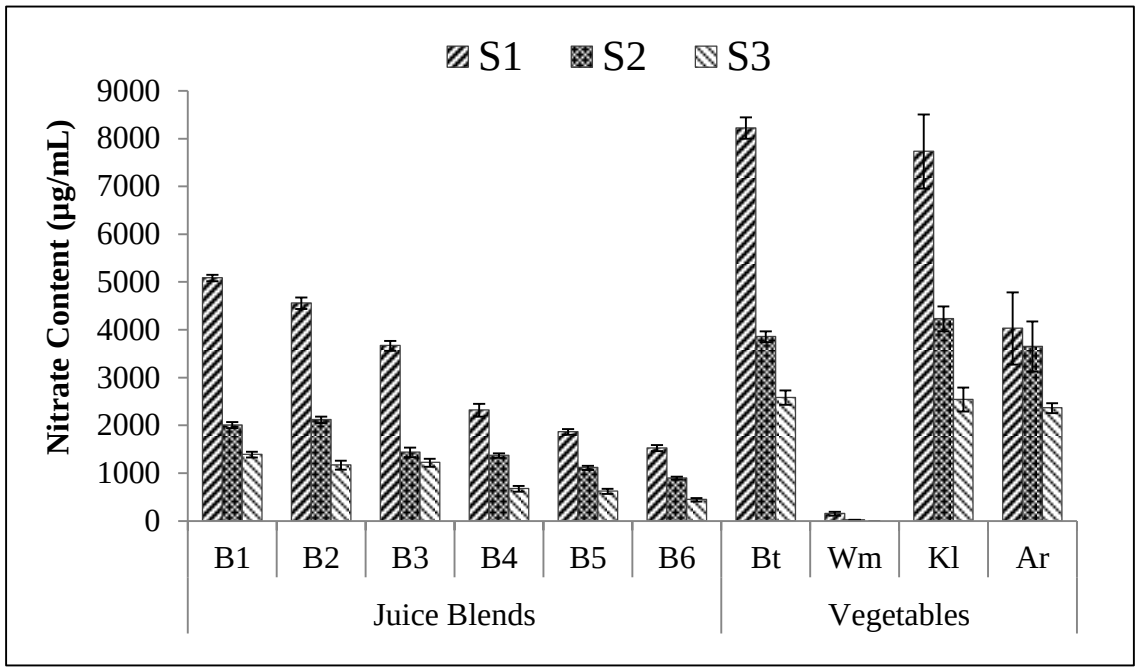

Figure A-1. Nitrate content measured by high pressure liquid chromatography for beetroot, watermelon, kale, arugula and blend juices from different stores. Abbreviations represent Bt-beetroot, Wm-watermelon, Kl-kale, Ar-arugula, B1-blend 1, B2-blend 2, B3-blend 3, B4-blend 4, B5- blend 5, and B6-blend. Different letters denote a significant difference between values at *$p \leq 0.05$ and **$p \leq 0.01$ ± Standard error calculated from 3 replicates for each store.

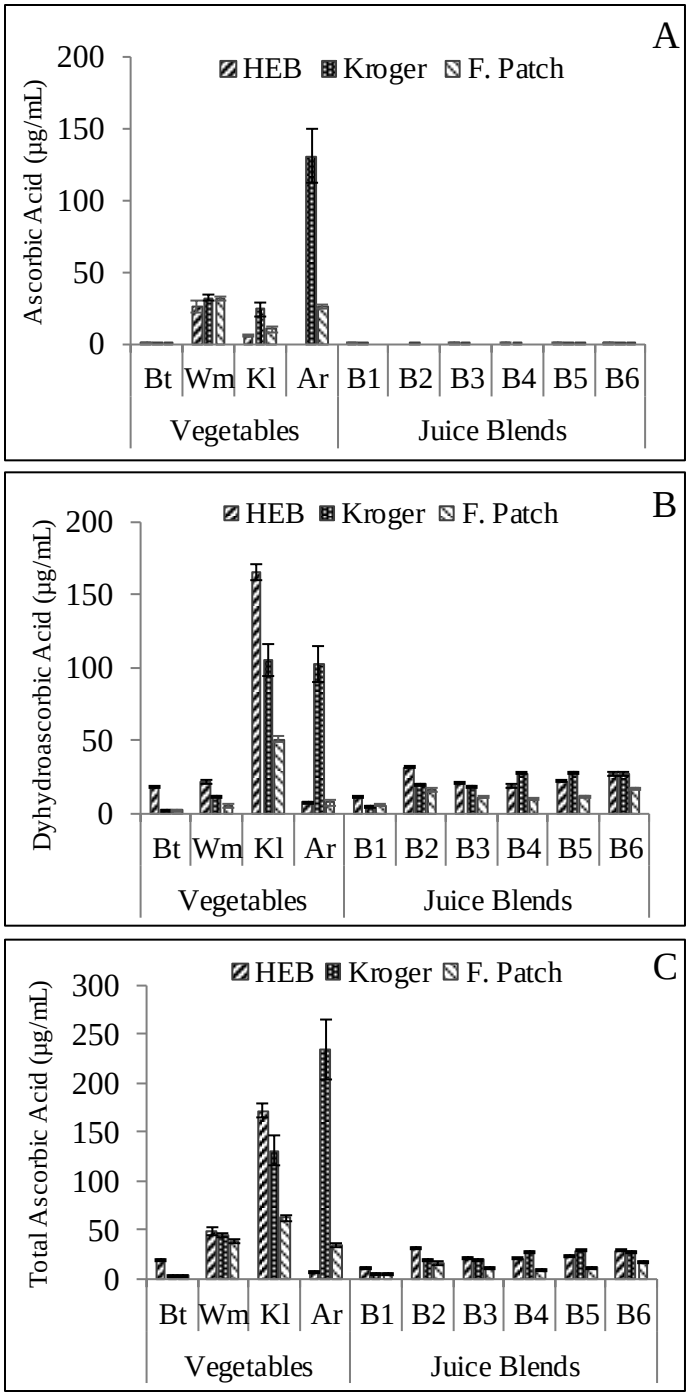

Figure A-2. Ascorbic Acid, dehydroascorbic acid, and total ascorbic content measured by HPLC for beetroot, watermelon, kale, arugula and blend juices from different stores. A) Ascorbic acid, B) dehydroascorbic acid, C) total ascorbic acid. Abbreviations represent Bt-beetroot, Wm-watermelon, Kl-kale, Ar-arugula, B1-blend 1, B2-blend 2, B3-blend 3, B4-blend 4, B5- blend 5, and B6-blend. Different letters denote a significant difference between values at *$p \leq 0.05$ and **$p \leq 0.01$ ± Standard error calculated from 3 replicates for each store.

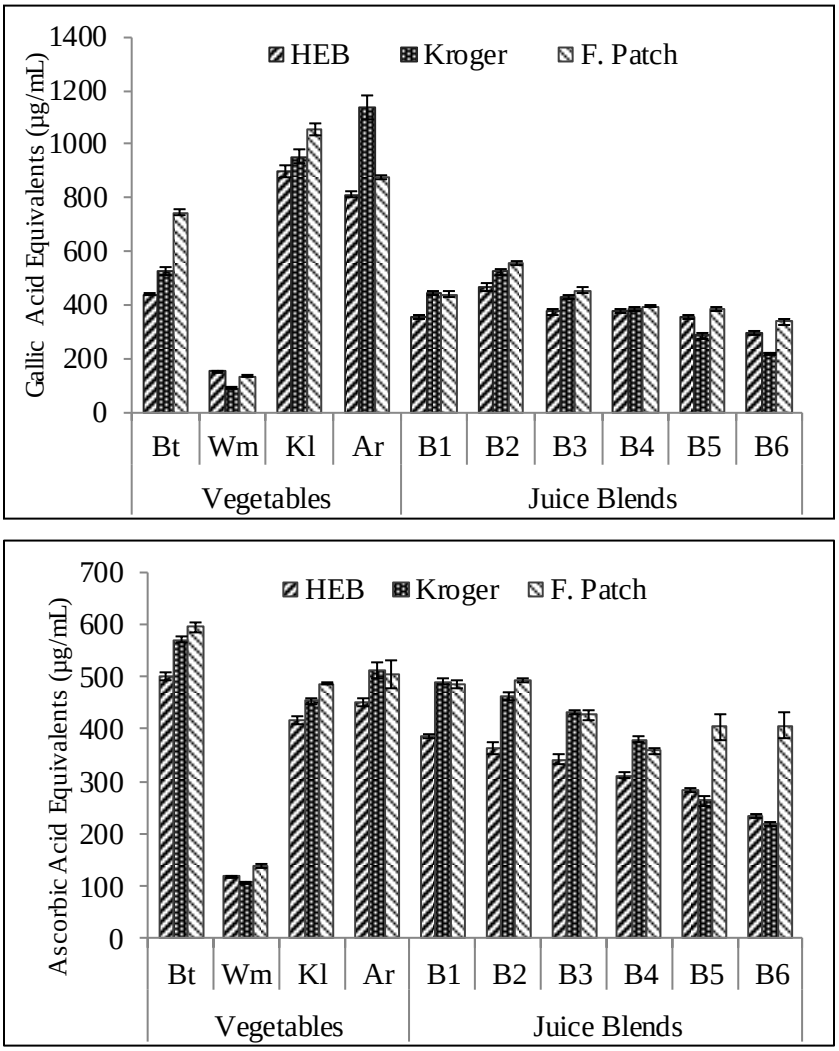

Figure A-3. Total phenolic content and DPPH radical scavenging activity in beetroot, watermelon, kale, arugula, and blend juices. A) Total phenolic content, B) DPPH radical scavenging activity. Abbreviations represent Bt-beetroot, Wm-watermelon, Kl-kale, Ar-arugula, B1-blend 1, B2-blend 2, B3-blend 3, B4-blend 4, B5- blend 5, and B6-blend. Different letters denote a significant difference between values at *$p \leq 0.05$ and **$p \leq 0.01$ ± Standard error calculated from 3 replicates for each store.

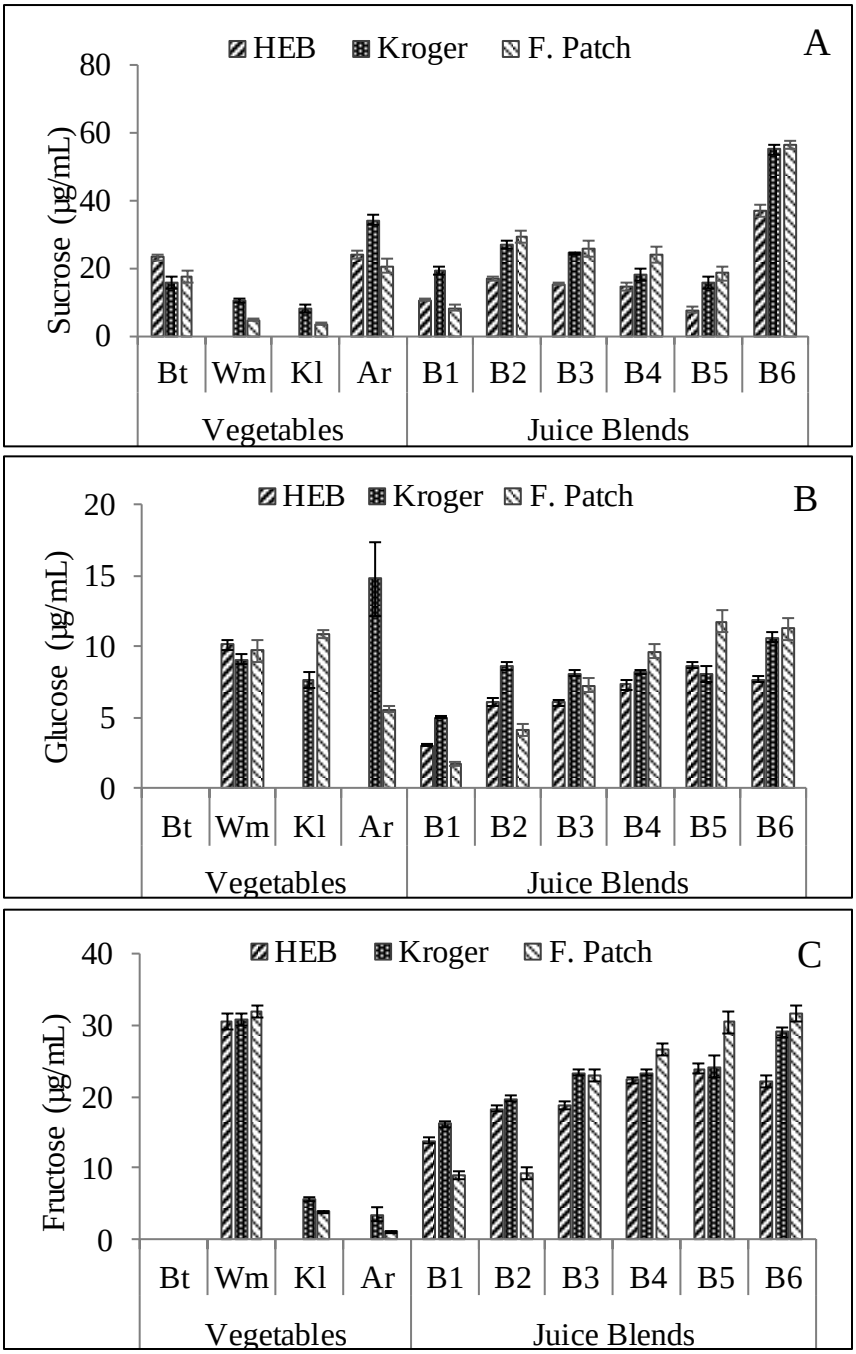

Figure A-4. Levels of sucrose (A), glucose (B), and fructose (C) in beetroot, watermelon, kale, arugula, and blend juices measured by high performance liquid chromatography. S Abbreviations represent Bt-beetroot, Wm-watermelon, Kl-kale, Ar-arugula, B1-blend 1, B2-blend 2, B3-blend 3, B4-blend 4, B5-blend 5, and B6-blend 6. Different letters denote a significant difference between values at *$p \leq 0.05$ and **$p \leq 0.01$ ± Standard error calculated from 3 replicates for each store.

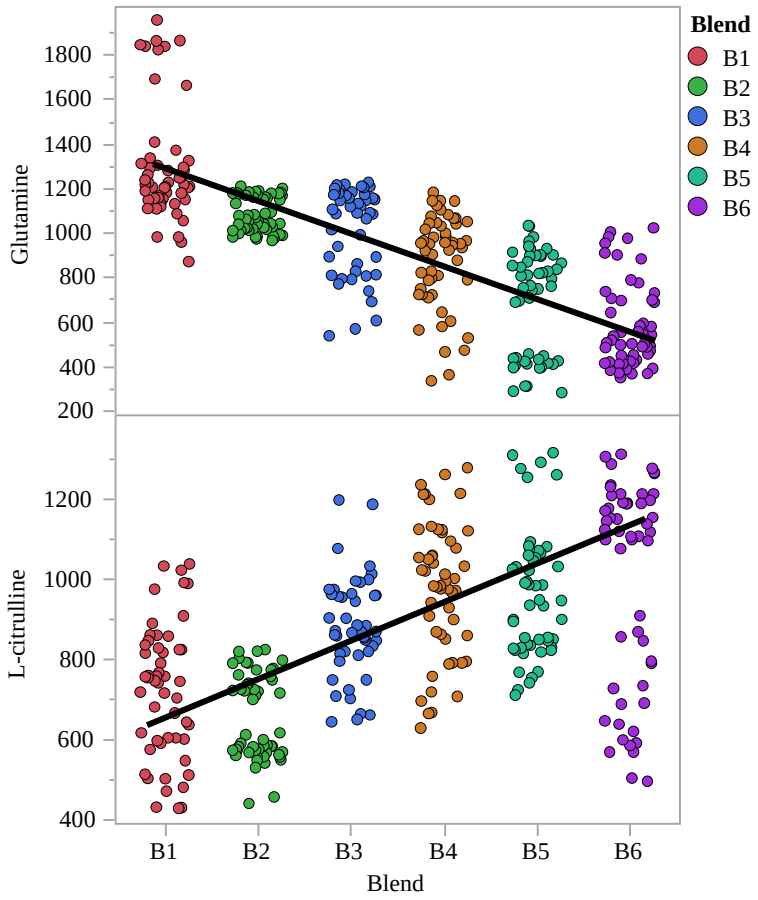

Figure A-5. The scatterplot matrix for glutamine and L-citrulline for the six juice blends. Abbreviations represent B1-blend 1, B2-blend 2, B3-blend 3, B4-blend 4, B5- blend 5, and B6-blend 6.

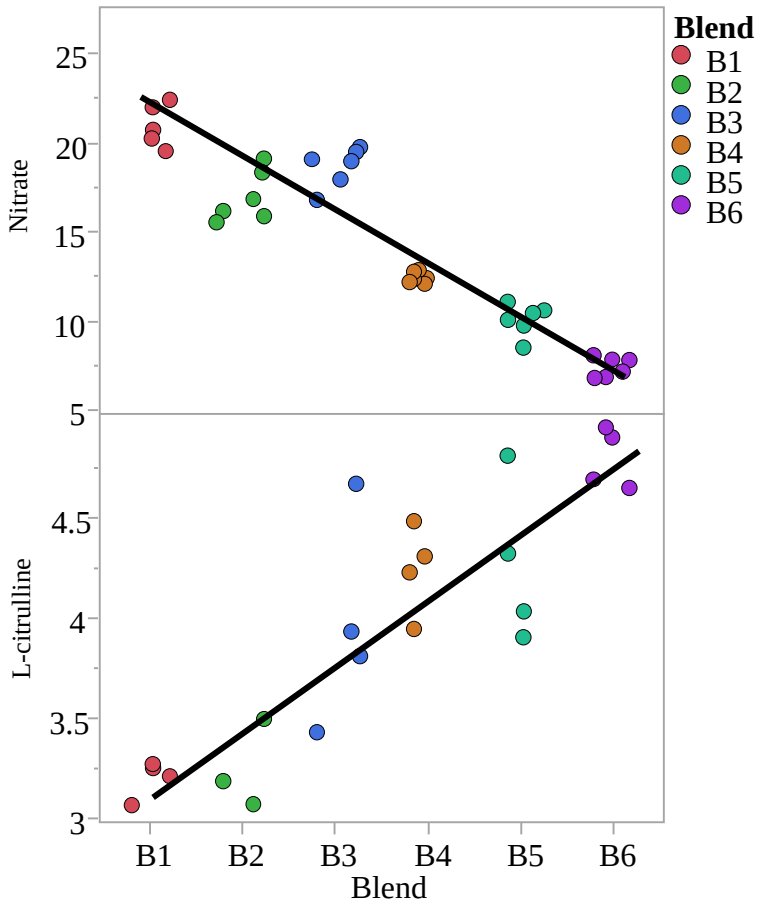

Figure A-6. The scatterplot matrix for nitrate and L-citrulline for the six freeze-dried juice blends. Abbreviations represent B1-blend 1, B2-blend 2, B3-blend 3, B4-blend 4, B5- blend 5, and B6-blend 6.

Table A-1 Effects of various column temperatures and 20 mM buffer at different pH on the separation of amino acids by HPLC-FLD represented in % recovery.

Analyte	20mM Buffer											
	pH 5.40				pH 5.60				pH 5.80			
	25˚C	30˚C	35˚C	40˚C	25˚C	30˚C	35˚C	40˚C	25˚C	30˚C	35˚C	40˚C
Asp	109.02	105.07	80.08	49.80	128.37	102.71	83.19	63.59	135.27	96.66	92.11	66.54
Glu	98.07	90.90	71.64	52.66	107.19	89.03	78.40	65.43	111.95	84.75	84.96	66.81
Asp	102.58	102.82	89.71	70.53	119.02	100.82	91.23	82.91	122.00	92.80	100.63	86.74
His	128.72	117.17	87.35	54.61	129.59	96.41	69.81	45.11	125.35	85.31	73.47	43.79
Ser	94.46	89.53	70.95	51.18	112.58	86.58	72.89	58.65	109.72	79.80	85.80	60.17
Gln	94.94	98.16	82.86	71.19	108.71	89.54	81.28	69.32	109.42	82.57	87.55	72.19
Cit	83.94	88.29	78.74	61.65	99.53	85.56	76.21	63.80	104.92	78.61	82.96	68.08
Arg	77.29	78.49	70.17	58.39	89.15	76.80	68.55	53.73	86.04	67.28	64.24	43.99
Gly	95.75	105.15	92.67	76.06	128.65	105.61	101.07	90.33	125.39	55.96	115.52	103.04
Thr	104.38	98.19	79.16	56.03	117.16	91.06	75.98	58.66	116.72	83.33	83.11	61.18
Ala	98.62	95.14	78.51	56.41	112.28	88.56	73.04	PM	110.24	82.54	PM	PM
β-ala	109.96	115.27	102.29	86.12	125.76	105.52	105.05	PM	127.43	63.38	PM	PM
Tyr	92.80	92.65	78.01	60.40	99.63	81.19	75.81	62.98	101.45	75.27	76.56	62.07
Met	76.91	85.89	77.41	62.78	80.42	77.66	74.52	66.98	76.28	66.61	79.92	68.93
Val	95.31	94.36	84.47	71.36	111.29	90.75	84.18	75.43	117.94	86.44	90.59	76.54
Trp	84.89	79.59	66.51	47.49	90.94	78.62	67.81	51.48	97.21	74.83	73.32	53.76
Phe	94.93	99.01	101.25	73.55	102.45	91.00	81.71	69.70	101.81	95.41	91.08	74.59
Iso	98.46	108.93	112.83	87.94	111.49	89.50	100.47	91.67	111.72	101.80	105.70	95.51
Leu	97.72	97.72	80.71	64.67	119.95	96.81	80.77	64.50	116.28	115.02	92.97	67.31
Orn	133.11	148.52	123.41	97.96	163.58	139.48	133.45	120.39	168.27	103.32	144.83	126.42
Lys	96.36	98.83	82.74	66.53	109.60	93.88	85.59	72.72	113.44	68.14	98.34	77.42

Values reported in % Recovery= (amount recovered/actual amount injected)*100. PM-peak merged.

Table A-2 Percent relative standard deviation (%RSD) for amino acids concentration and retention times in standard mixture and watermelon juice samples.

Analyte	Concentration % RSD (n=5)				Retention Time % RSD (n=5)			
	Watermelon		Standard		Watermelon		Standard	
	Intra-day	Inter-day	Intra-day	Inter-day	Intra-day	Inter-day	Intra-day	Inter-day
Asp	6.66	5.51	1.03	2.08	0.91	3.66	1.26	5.03
Glu	2.54	3.63	1.26	2.31	1.10	4.16	1.04	2.95
Asn	4.25	2.04	1.41	3.04	0.49	1.83	0.93	1.14
His	4.44	2.96	1.23	5.41	0.50	1.50	1.01	0.96
Ser	4.75	1.78	0.86	2.83	0.38	1.54	0.80	0.99
Gln	5.47	1.65	0.99	3.64	0.36	1.51	0.62	0.87
Cit	2.92	3.50	1.34	1.15	0.50	1.55	0.49	0.49
Arg	3.85	1.72	2.15	10.76	0.47	1.58	0.44	0.29
Gly	ND	ND	2.41	2.35	ND	ND	0.41	0.46
Thr	5.93	3.63	0.40	3.71	0.45	1.67	0.36	0.44
Ala	6.92	2.25	1.48	4.09	0.36	1.93	0.50	0.20
β-ala	1.75	2.55	1.34	2.73	0.58	2.01	0.59	0.35
Tyr	3.98	2.38	2.62	2.26	0.40	2.14	0.66	0.15
Met	5.82	1.16	1.16	5.49	0.21	0.90	0.29	0.90
Val	3.51	3.06	1.05	5.62	0.20	0.95	0.27	0.99
Trp	5.17	3.04	0.59	5.39	0.17	0.98	0.26	0.94
Phe	5.32	3.08	0.73	3.41	0.22	1.29	0.30	0.98
Iso	5.63	4.62	0.84	4.84	0.23	1.45	0.30	1.01
Leu	4.33	4.79	0.88	1.75	0.23	1.52	0.44	0.97
Orn	3.41	1.92	1.19	1.87	0.12	2.19	0.34	1.00
Lys	5.46	4.63	1.26	1.97	0.13	1.81	0.35	1.01

For precision analysis 5 injections (intraday) were performed within a day for 3 consecutive days (interday). ND not detected. RSD (%) = relative standard deviation; (standard deviation/mean)×10

Table A-3. Total soluble solids, pH and % titratable acidity for beetroot, watermelon, kale arugula, and blend juices from three different stores.

Juice Samples	TSS			pH			%TA		
	HEB	Kroger	F. Patch	HEB	Kroger	F. Patch	HEB	Kroger	F. Patch
Beet	9.18 ±0.07	10.42 ±0.21	11.84 ±0.12	6.26 ±0.01	6.33 ±0.01	6.18 ±0.00	0.14 ±0.00	0.12 ±0.00	0.13 ±0.00
Watermelon	9.23 ±0.09	9.13 ±0.24	8.74 ±0.10	6.10 ±0.02	5.78 ±0.02	5.59 ±0.07	0.11 ±0.00	0.13 ±0.00	0.10 ±0.00
Kale	7.31 ±0.03	7.02 ±0.24	6.97 ±0.08	5.95 ±0.01	6.11 ±0.00	5.85 ±0.01	0.28 ±0.00	0.20 ±0.01	0.21 ±0.00
Arugula	5.72 ±0.04	6.61 ±0.45	4.54 ±0.02	6.35 ±0.01	6.15 ±0.01	6.03 ±0.00	0.18 ±0.00	0.17 ±0.00	0.15 ±0.00
Blend 1	9.13 ±0.04	9.86 ±0.09	10.01 ±0.12	6.11 ±0.02	5.99 ±0.02	6.76 ±0.03	0.13 ±0.00	0.13 ±0.00	0.13 ±0.01
Blend 2	8.67 ±0.07	8.99 ±0.07	8.90 ±0.09	6.14 ±0.01	6.09 ±0.01	6.73 ±0.02	0.15 ±0.00	0.13 ±0.00	0.13 ±0.00
Blend 3	8.80 ±0.04	9.21 ±0.10	9.02 ±0.11	6.19 ±0.02	6.02 ±0.01	6.05 ±0.03	0.14 ±0.00	0.13 ±0.00	0.12 ±0.00
Blend 4	8.93 ±0.11	9.06 ±0.14	8.79 ±0.10	6.22 ±0.03	5.98 ±0.02	5.97 ±0.04	0.13 ±0.00	0.13 ±0.00	0.12 ±0.00
Blend 5	8.84 ±0.11	9.08 ±0.17	8.71 ±0.11	6.19 ±0.02	5.96 ±0.01	5.88 ±0.04	0.13 ±0.00	0.13 ±0.00	0.12 ±0.00
Blend 6	8.94 ±0.10	8.98 ±0.18	8.58 ±0.12	6.16 ±0.02	5.90 ±0.01	5.83 ±0.06	0.13 ±0.00	0.12 ±0.01	0.11 ±0.00

TSS= Total soluble solids, represented in (Brix^{o}). % TA= Percent titratable acidity, represented in citric acid equivalents. Different letters denote a significant difference between values at *$p \leq 0.05$ and **$p \leq 0.01$ ± Standard error calculated from 3 replicates for each store.

Table A-4 L*, a, b*, C*, and h color values for beetroot, watermelon, kale, arugula and blend juices.

Juices	L*	a*	b*	C*	h
Beet	24.36 ±0.13	2.21 ±0.07	-1.47 ±0.02	2.71 ±0.06	325.84 ±1.09
Watermelon	32.35 ±0.15	19.63 ±0.40	6.58 ±0.15	20.74 ±0.41	18.90 ±0.19
Kale	30.86 ±0.39	-7.35 ±0.18	7.69 ±0.21	10.64 ±0.26	133.72 ±0.43
Arugula	31.27 ±0.29	-7.36 ±0.27	8.14 ±0.39	10.99 ±0.46	132.38 ±0.47
B1	23.97 ±0.23	3.04 ±0.04	-1.30 ±0.02	3.31 ±0.04	336.77 ±0.43
B2	25.12 ±0.12	3.30 ±0.07	-0.88 ±0.07	3.44 ±0.06	343.59 ±1.52
B3	25.20 ±0.11	3.74 ±0.08	-1.00 ±0.07	3.89 ±0.07	344.91 ±1.40
B4	25.40 ±0.16	4.10 ±0.12	-0.73 ±0.10	4.20 ±0.11	308.99 ±21.40
B5	25.56 ±0.12	3.95 ±0.10	-0.60 ±0.10	4.04 ±0.09	310.59 ±21.13
B6	25.99 ±0.14	4.14 ±0.15	-0.26 ±0.13	4.21 ±0.15	234.89 ±31.63

Different letters denote a significant difference between values at *$p \leq 0.05$. C*-chroma and h- hue. Different letters denote a significant difference between values at *$p \leq 0.05$. Samples expressed in mean of 9 replicates (3 replications per store) ± SE.

Table A-5. L*, a, b*, C*, and h color values for beetroot, watermelon, kale, arugula and blend juices from different stores.

Juices	S-1										S-2										S-3									
	L*		a*		b*		C*		h		L*		a*		b*		C*		h		L*		a*		b*		C*		h	
B1	22.79	±0.17	2.89	±0.06	-1.24	±0.03	3.15	±0.05	336.65	±0.93	24.10	±0.33	3.05	±0.07	-1.36	±0.01	3.34	±0.06	335.85	±0.55	25.03	±0.26	3.18	±0.05	-1.30	±0.03	3.44	±0.04	337.81	±0.64
B2	24.96	±0.14	3.72	±0.07	-0.42	±0.02	3.74	±0.07	350.58	±3.14	25.35	±0.24	2.92	±0.08	-1.25	±0.02	3.18	±0.07	336.83	±0.78	25.05	±0.21	3.26	±0.04	-0.97	±0.03	3.40	±0.04	343.36	±0.49
B3	24.71	±0.11	4.16	±0.08	-0.53	±0.04	4.20	±0.08	353.55	±2.44	25.28	±0.15	3.31	±0.11	-1.31	±0.02	3.56	±0.10	338.23	±0.80	25.61	±0.17	3.74	±0.08	-1.15	±0.04	3.92	±0.09	342.96	±0.30
B4	25.35	±0.28	4.83	±0.17	-0.13	±0.02	4.83	±0.17	358.39	±0.20	25.40	±0.22	3.61	±0.09	-1.13	±0.03	3.79	±0.08	342.52	±0.99	25.45	±0.33	3.86	±0.06	-0.97	±0.04	3.98	±0.05	345.81	±0.68
B5	25.61	±0.10	4.54	±0.09	-0.13	±0.01	4.55	±0.09	358.28	±0.13	25.77	±0.27	3.74	±0.14	-1.00	±0.04	3.87	±0.14	344.12	±0.29	25.31	±0.19	3.58	±0.04	-0.94	±0.03	3.70	±0.03	345.28	±0.51
B6	26.39	±0.18	5.13	±0.15	0.63	±0.09	5.18	±0.15	6.85	±0.83	25.73	±0.29	3.64	±0.07	-0.74	±0.03	3.71	±0.07	348.51	±0.53	25.86	±0.21	3.66	±0.05	-0.69	±0.03	3.73	±0.05	349.31	±0.58
Bt	24.37	±0.12	1.87	±0.01	-1.56	±0.02	2.44	±0.02	320.17	±0.35	24.22	±0.27	2.13	±0.03	-1.52	±0.01	2.73	±0.11	324.43	±0.26	24.50	±0.29	2.63	±0.10	-1.34	±0.01	2.96	±0.09	332.90	±0.90
Wm	32.82	±0.14	21.78	±0.34	7.17	±0.09	22.93	±0.35	18.24	±0.07	32.45	±0.28	17.35	±0.26	6.03	±0.31	18.48	±0.30	20.11	±0.26	31.77	±0.21	19.77	±0.41	6.55	±0.14	20.82	±0.43	18.34	±0.03
Kl	30.85	±1.17	-8.37	±0.06	9.05	±0.15	12.33	±0.15	132.77	±0.27	30.93	±0.07	-7.39	±0.08	6.98	±0.11	10.16	±0.13	136.65	±0.14	30.79	±0.29	-6.28	±0.13	7.04	±0.17	9.44	±0.21	131.75	±0.21
Ar	32.86	±0.28	-8.80	±0.27	10.40	±0.58	13.64	±0.62	130.49	±0.69	30.98	±0.28	-7.60	±0.11	7.61	±0.17	10.77	±0.20	135.01	±0.31	29.96	±0.31	-5.67	±0.07	6.39	±0.16	8.55	±0.16	131.65	±0.40

Abbreviations represent Bt-beetroot, Wm-watermelon, Kl-kale, Ar-arugula, B1-blend 1, B2-blend 2, B3-blend 3, B4-blend 4, B5- blend 5, and B6-blend. S-1= HEB, S-2= Kroger, and S-3= Farm Patch. Means± Standard error calculated from 3 replicates.

Table A-6. Total color difference ΔE* for beetroot, watermelon, kale, arugula and blend juices.

Juice Blends	Vegetable Juice																					
	Beet						Watermelon						Kale						Arugula					
	S-1		S-2		S-3		S-1		S-2		S-3		S-1		S-2		S-3		S-1		S-2		S-3	
Blend 1	1.94	±0.09	1.49	±0.19	1.05	±0.21	22.99	±0.27	18.16	±0.40	19.56	±0.43	17.54	±0.38	15.04	±0.15	13.92	±0.36	19.35	±0.61	15.58	±0.34	12.78	±0.27
Blend 2	2.30	±0.10	1.69	±0.25	1.18	±0.30	21.12	±0.40	17.68	±0.34	19.36	±0.48	16.83	±0.25	14.34	±0.23	13.73	±0.32	18.36	±0.60	14.90	±0.35	12.60	±0.27
Blend 3	2.55	±0.10	1.79	±0.23	1.70	±0.27	20.87	±0.29	17.41	±0.38	18.82	±0.46	17.27	±0.25	14.68	±0.18	13.97	±0.31	18.83	±0.65	15.23	±0.30	12.86	±0.25
Blend 4	3.55	±0.26	2.02	±0.19	1.75	±0.17	19.90	±0.50	17.08	±0.33	18.70	±0.43	17.30	±0.20	14.77	±0.23	14.01	±0.27	18.81	±0.64	15.31	±0.31	12.91	±0.22
Blend 5	3.39	±0.14	2.41	±0.33	1.55	±0.29	19.99	±0.25	16.74	±0.45	18.99	±0.48	16.88	±0.24	14.64	±0.18	13.84	±0.30	18.37	±0.58	15.15	±0.28	12.72	±0.24
Blend 6	4.45	±0.13	2.40	±0.26	1.93	±0.24	19.01	±0.33	16.74	±0.31	18.62	±0.42	16.90	±0.25	14.45	±0.24	13.55	±0.29	18.24	±0.60	14.99	±0.31	12.44	±0.23

S-1= HEB, S-2= Kroger, and S-3= Farm Patch ± Standard error calculated from 3 replicates.